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STUDY PROTOCOL OF A PHASE IB/II CLINICAL TRIAL OF METFORMIN AND CHLOROQUINE IN PATIENTS WITH *IDH1*-MUTATED OR *IDH2*-MUTATED SOLID TUMOURS

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ABSTRACT

Introduction: High-grade chondrosarcoma, high-grade glioma, and intrahepatic cholangiocarcinoma are aggressive types of cancer with a dismal outcome. This is due to the lack of effective treatment options, emphasizing the need for novel therapies. Mutations in the genes *IDH1* and *IDH2* occur in 60% of chondrosarcoma, 80% of WHO grade II-IV glioma and 20% of intrahepatic cholangiocarcinoma. *IDH1/2*-mutated cancer cells produce the oncometabolite *D*-2-hydroxyglutarate (*D*-2HG) and are metabolically vulnerable to treatment with the oral antidiabetic metformin and the oral antimalarial drug chloroquine.

Methods and analysis: We describe a dose-finding phase Ib/II clinical trial, in which patients with *IDH1/2*-mutated chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma are treated with a combination of metformin and chloroquine. Dose escalation is performed according to a 3+3 dose-escalation scheme. The primary objective is to determine the maximum tolerated dose to establish the recommended dose for a phase II clinical trial. Secondary objectives of the study include (1) determination of pharmacokinetics and toxic effects of the study therapy, for which metformin and chloroquine serum levels will be determined over time; (2) investigation of tumour responses to metformin plus chloroquine in *IDH1/2*-mutated cancers using CT/MRI scans; and (3) whether or not tumour responses can be measured by non-invasive *D*-2HG measurements (mass spectrometry (MS) and magnetic resonance spectroscopy (MRS)) of tumour tissue, serum, urine, and/or bile or next-generation sequencing of circulating tumour DNA (liquid biopsies).

Ethics and dissemination: This study has been approved by the local medical-ethical review committee. Results and data will be submitted to a peer-reviewed journal.

Conclusion: This study may open a novel treatment avenue for *IDH1/2*-mutated high-grade chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma by repurposing the combination of two inexpensive drugs that are already approved for other indications.

STRENGTHS AND LIMITATIONS OF THIS STUDY

Strengths:

- Metformin and chloroquine have shown synergistic anti-tumour effects on *IDH1/2*-mutated cancer cells.
- Metformin and chloroquine are two safe, inexpensive drugs that are already approved for other indications.
- Our study investigates non-invasive markers to assess tumour responses to therapy.

Limitations:

- Because this is primarily a dose-finding study, we may not be able to study the efficacy of metformin and chloroquine.
- When patients do not consent to tumour biopsies/re-resections, this diminishes the possibility for translational analyses.

ARTICLE MANUSCRIPT

Introduction

IDH1 and IDH2 are homodimeric enzymes that reversibly convert isocitrate to α-ketoglutarate (αKG) in cytoplasm and mitochondria, respectively. Somatic heterozygous mutations in *IDH1/2* that produce the oncometabolite *D*-2-hydroxyglutarate (*D*-2HG) are observed in substantial percentages of various tumour types such as chondrosarcoma (60%), WHO grade II-III glioma (80%), secondary WHO grade IV glioblastoma (80%), and intrahepatic cholangiocarcinoma (20%).[1] In addition, *IDH1/2* mutations occur in varying percentages of acute lymphocytic leukaemia (10%), acute myeloid leukaemia (AML; 20%), angioimmunoblastic T-cell lymphoma (40%), colorectal cancer (5%), and melanoma (12%).[1] In chondrosarcoma and glioma, *IDH1/2* mutations are considered very early or even inaugural genetic defects, and are thus present in a large fraction of, or even all, cancer cells.[2 3] This renders *IDH1/2* mutations an interesting target for anti-cancer treatment because such tumour homogeneity decreases the risk of therapy resistance.[4] Recently, inhibitors of mutant IDH1 and IDH2 were developed that may be effective in stalling malignant progression of early-stage *IDH1/2*-mutated cancers.[5 6]

Prognosis and therapeutic options of cancers in which *IDH1/2* mutations occur

The prognosis of solid tumours with frequent occurrence of *IDH1/2* mutations remains poor. The current standard therapy for chrondrosarcoma is surgery. There is no evidence for a benefit of (adjuvant) radiotherapy or chemotherapy, as chondrosarcoma are considered to be highly therapy resistant.[7] Consequently, the 1-year survival rate of metastasized high-grade chondrosarcoma is <10%.[8]

Gliomas vary from WHO grade II diffuse astrocytoma and diffuse oligodendroglioma, with median survivals of more than five years,[9] to WHO grade IV glioblastoma, with a median survival of only 15 months despite aggressive treatment using radiotherapy and temozolomide.[10] Gliomas are diffusely growing tumours, which renders surgery ineffective, emphasizing the dire need for novel therapies. Furthermore, the blood-brain barrier (BBB) prohibits the use of most chemotherapeutics and the surrounding normal brain hampers aggressive radiotherapy regimens due to limitations that are raised by healthy brain tissue.[11]

Intrahepatic cholangiocarcinoma is resectable in only 40% of patients.[12] In unresectable cases, intrahepatic cholangiocarcinoma patients are offered palliative treatment as standard of care with the chemotherapy combination of cisplatin and gemcitabine, with a median overall survival of 11.7 months.[13]

Metabolic effects of *IDH1/2* mutations

Heterozygous hotspot *IDH1/2* mutations disable IDH1/2 wild-type enzyme activity[14-16] and induce a neo-enzymatic activity that leads to the production and subsequent accumulation of *D*-2HG.[17-19] *D*-2HG is normally present only in trace amounts in normal tissues and cells but accumulates up to 50 mM in *IDH1/2*-mutated glioma.[17] *D*-2HG is chemically very similar to α-ketoglutarate (αKG) and inhibits over 60 αKG-dependent enzymes, resulting in global DNA/histone hypermethylation, decreased hypoxia-inducible factor 1a (HIF1α) expression, and perturbed collagen maturation.[1] Depending on the cellular context, these effects are the basis of oncogenesis and imply a dependence on *D*-2HG of early-stage *IDH1/2*-mutated tumours.[1]

IDH1/2-mutated cancer cells need αKG to synthesize *D*-2HG and fuel the tricarboxylic acid (TCA) cycle to support their metabolism. αKG is generated by glycolysis (glucose breakdown) or glutaminolysis (glutamine/glutamate breakdown).[20] *IDH1/2* mutations downregulate αKG levels by consuming αKG and by inhibition of αKG production via direct effects, *i.e.* by disabling *IDH1/2* wild-type kinetics, and indirect effects, *e.g.* by decreasing TCA cycle activity.[1] Therefore, *IDH1/2*-mutated cancer cells rely on glutaminolysis for sufficient αKG supply to generate the oncometabolite *D*-2HG (**Figure 1**).[21] The conversion of glutamate to αKG is catalysed by glutamate dehydrogenase (GDH), which is the final step of glutaminolysis and can be inhibited by the anti-malaria drug chloroquine and the antidiabetic drug metformin.[20 22-24] In addition, *IDH1*-mutated glioma cells show increased levels of autophagy, likely as a survival mechanism of cells to metabolic stress by catabolizing proteins in order to provide substrates for energy production in stress/starvation contexts.[25] Autophagy is inhibited by chloroquine[26] and the anti-cancer properties of chloroquine may thus be selective for *IDH1/2*-mutated cells because it inhibits glutaminolysis and autophagy on which the cells are dependent.

In addition to a dependence on glutaminolysis and autophagy, *IDH1/2* mutations induce further metabolic stress in *IDH1/2*-mutated cancer cells via inhibition of the TCA cycle and electron transport chain (ETC) by *D*-2HG. More specifically, *D*-2HG inhibits enzymatic activity of complex IV (cytochrome C oxidase) of the ETC[27] and the TCA(-like) enzymes IDH1/2 and αKG dehydrogenase.[16] This reduces oxidative phosphorylation, the primary source of ATP in cancer cells.[27 28] This metabolic stress is amplified *in vitro* in glioma and colorectal carcinoma cells using compounds that inhibit ETC complex I, such as the oral antidiabetic biguanides metformin and phenformin.[16 28] Furthermore, the inhibition of ETC

complex IV activity by *D*-2HG lowers the mitochondrial threshold to trigger apoptosis in AML cells and this can be therapeutically exploited by pharmacological BCL-2 inhibition.[27] These inhibitors induce selective growth rate reduction and/or apoptosis in various types of *IDH1*-mutated glioma, colorectal carcinoma, and AML cells, but not in *IDH1/2* wild-type counterparts.

Metabolism of *IDH1/2*-mutated tumours as therapeutic target

Metformin and chloroquine increase metabolic stress in *IDH1/2*-mutated cells, as is described above. Patients with *IDH1/2*-mutated glioblastoma have a prolonged survival and better radiotherapy/chemotherapy response when compared with *IDH1/2* wild-type counterparts,[14 29 30] while in chondrosarcoma a correlation between mutation and survival was absent.[2] We and others have shown that *IDH1/2* mutations sensitize glioma and colorectal carcinoma cells to therapies that involve oxidative stress, such as radiotherapy, cisplatin, and carmustine.[16 31] Combined, these data suggest that at least some types of cancer with *IDH1/2* mutations should be targeted by compounds that exploit this presumed metabolic vulnerability rather than compounds that decrease metabolic stress (*i.e.* IDH1/2-mutant inhibitors). Accordingly, we hypothesized that the difference in survival of patients with *IDH1/2*-mutated glioma or intrahepatic cholangiocarcinoma versus patients with *IDH1/2* wild-type counterparts are caused by dysregulation of cellular defence mechanisms by *IDH1/2* mutations against anti-cancer therapy.[1 16 32] Little is known about the role of *IDH1/2* mutations in late-stage cancer. It is plausible that with increasing mutational burden, the dependence of late-stage malignant tumours on *IDH1/2* mutations decreases, diminishing the therapeutic index of IDH1/2-mutant inhibitors.[33 34] On the other hand, metabolic stress that results from *IDH1/2* mutations persists, and this metabolic vulnerability provides an excellent target for therapy irrespective of the tumour stage.

Discussion regarding the use of metformin and chloroquine

Metformin and chloroquine are readily-available, inexpensive, and safe drugs that are already FDA/EMA-approved for other indications. The safety profiles of metformin and chloroquine are favourable over other anti-cancer modalities, which may aid rapid implementation of these drugs into therapies for patients with *IDH1/2*-mutated cancers. A caveat is that the combined safety of metformin and chloroquine is to be proven by our study, although there are no reports of toxic side-effects of this combination in the literature whereas the prevalence of both diabetes and malaria is high. Since both drugs are off patent, metformin and chloroquine can become a therapeutic advance for patients with *IDH1/2*-mutated solid tumours that is considerably less expensive than products of other anti-cancer research efforts. The potential of metformin and chloroquine as adjuvant drugs was recently demonstrated *in vivo,* where metformin or chloroquine had a sensitizing and/or synergistic anti-tumour effect in combination with temozolomide,[35 36] cisplatin,[37 38] and gemcitabine[39 40] in xenograft models or proof-of-concept clinical trials of various types of human cancer, including glioma. Metformin, but not chloroquine, sensitized xenograft models of various types human cancer to ionizing radiation.[41 42]

Possible concerns may be related to the bioavailability of metformin. We have observed high expression of metformin transporters in chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma cell lines and primary tissue (OCT1-3; The Cancer Cell Line Encylopedia and our own unpublished data). Therefore, we expect to achieve sufficient intratumoural metformin concentrations with dose levels 2 and 3 of our dose-escalation protocol. Whereas millimolar metformin concentrations are necessary to activate the necessary antineoplastic cellular targets *in vitro,* these targets were already activated at

±300-fold lower metformin concentrations *in vivo*. When metformin fails to show any metabolic or anti-tumour effect, we will investigate the feasibility of using phenformin, which is the lipophilic analogue of metformin which does not depend on transporters to enter cells. In concordance with the latter statement, phenformin has shown a better bioavailability than metformin in patients.[43] A disadvantage of phenformin compared with metformin is the increased risk of lactic acidosis. As a consequence, phenformin approval for the treatment of diabetes mellitus type 2 was withdrawn by the FDA and EMA in the 1970s.[43] However, the increased risk of lactic acidosis may be more acceptable in the setting of cancer treatment.

With respect to chloroquine, possible concerns may be related to the plethora of cellular targets that chloroquine affects. Besides inhibition of autophagy and glutaminolysis, the two potential therapeutic targets of chloroquine in *IDH1/2*-mutated cancers, chloroquine also contributes to the induction of apoptosis, buffers the tumour milieu, and affects the body's immune response to the tumour *in vitro* and/or *in vivo* at concentrations that may be achieved using the dose that we use in the present clinical trial.[44] These properties of chloroquine as a "dirty drug" may lead to non-specific anti-tumour effects or toxicity problems in the treatment of *IDH1/2*-mutated solid tumours. Conversely, the buffering of the tumour milieu may aid the efficacy of chloroquine in the treatment of *IDH1/2*-mutated glioma, which grow very diffusely and for which it was hypothesized that this is due to an increased acidification of *IDH1/2*-mutated glioma cells of their microenvironment due to excess deposition of the acidic *D*-2HG.[45 46] Chloroquine may decrease this acidification, reduce the diffuse growth and ultimately increase the treatability of *IDH1/2*-mutated glioma.

For the treatment of glioma, adequate drug penetration of the BBB is necessary for relevant tumour responses. Notwithstanding that high-grade glioma often destruct the BBB, *in vivo*

experiments in mice have shown that metformin and chloroquine adequately pass the blood-brain barrier (**ref Pharmacol Rep 2010;62:956-65 and PLoS One 2012;7:e47357**).

Non-invasive detection of *IDH1/2* mutations

The gold standard of *IDH1/2* mutation detection is genetic analysis of tumour DNA. In glioma, 90% of all *IDH1/2* mutations are $IDH1^{R132H}$ and its presence can be reliably detected using a immunohistochemistry of glioma tissue with an $IDH1^{R132H}$-specific antibody.[47] The presence of *IDH1/2* mutations in AML[48] and intrahepatic cholangiocarcinoma[49] can be easily, reliably, and non-invasively detected via determination of 2HG levels or *D*-2HG levels in serum or urine by mass spectrometry (MS). Furthermore, MS-determined 2HG serum levels correlate with therapy response in these cancers.[48 49] In a previous study investigating intrahepatic cholangiocarcinoma, total 2HG levels in serum predicted the presence of an *IDH1/2* mutation (as determined using targeted DNA sequencing) with a sensitivity of 83% and a specificity of 90%.[49]

Whereas no non-invasive detection methods of *IDH1/2* mutations have been described to be effective in chondrosarcoma yet, the presence of *IDH1/2* mutations in glioma can be determined using magnetic resonance spectroscopy (MRS) of the brain, which detects intratumoural 2HG levels.[50 51] Conversely, serum 2HG levels correlate poorly with the *IDH1/2* mutational status in glioma due to a limited blood-brain barrier passage of *D*-2HG.[52] Urine 2HG levels are higher in patients with *IDH1*-mutated glioma than in patients with *IDH1* wild-type glioma,[53] although another study reported decreased 2HG levels in the urine of patients with *IDH1*-mutated glioma and showed that the ratio of serum 2HG levels to urine 2HG levels is most predictive for the *IDH1* mutational status in glioma.[54] Most aforementioned

measurements determined total 2HG levels and thus did not discriminate between the *D*-enantiomer of 2HG (which is specific for *IDH1/2* mutations) and the *L*-enantiomer of 2HG (which is unspecific and is generated during hypoxia).[55 56] Better separation of *D*-2HG and *L*-2HG may allow for *IDH1/2* mutational status predictions with higher sensitivity and specificity.

Besides methods that detect *D*-2HG accumulation, *IDH1/2* mutations may also be detected via next-generation sequencing (NGS) of circulating tumour DNA (ctDNA) that is isolated from serum as liquid biopsies. Liquid biopsies contain a collection of ctDNA sequences which is representative for the heterogeneity of the tumour. Therefore, liquid biopsies are more informative than tissue biopsies, which are subject to selection bias as a result of the tumour heterogeneity. In liquid biopsies, variant allelic frequencies can be used as biomarkers for tumour load and dynamic clonal hierarchies within the tumour.[57]

Hypothesis and outlook

To summarize, fundamental and translational research by us and others revealed that *IDH1/2* mutations impart therapeutically targetable metabolic vulnerabilities to cells from several types of cancer.[16 20 21 27 28] We aim to use these metabolic alterations in *IDH1/2*-mutated tumours for screening purposes and tumour response monitoring purposes using non-invasive modalities. Furthermore, we aim to specifically inhibit the metabolic processes that are essential to *IDH1/2*-mutated tumours using metformin and chloroquine, which specifically target the metabolic vulnerabilities that are caused by *IDH1/2* mutations.

We hypothesize that metformin and chloroquine can be safely used as anti-cancer drugs for patients with *IDH1/2*-mutated chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma and that tumour response to treatment can be monitored by measuring tumour size and/or

levels of *D*-2HG in serum, urine, bile, and/or the tumoural mass. This hypothesis will be tested in a phase Ib/II clinical trial. There are no reports of clinical trials of combined treatment with metformin and chloroquine yet. In the future, metformin and chloroquine may be used as stand-alone therapy for patients with *IDH1/2*-mutated cancers, especially in chondrosarcoma for which no effective therapies beside surgery exists, or besides conventional anti-cancer treatments such as radiation and temozolomide in glioma and cisplatin and gemcitabine in intrahepatic cholangiocarcinoma.

Methods and analysis

Overall study design

MACIST is a nonrandomized, open-label, dose-finding, multi-centre phase Ib/II clinical trial with a combined regimen of metformin and chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma. Drug dosing will follow a 3+3 dose escalation scheme. Patients will be enrolled at three sites in The Netherlands.

Objectives

Primary objective

To determine the maximum tolerated dose (MTD) and recommended dose (RD) of metformin plus chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma.

Secondary objectives

- To describe the toxic effects and pharmacokinetics of metformin plus chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma;
- To provide evidence of complete or partial tumour regression in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma after treatment with metformin plus chloroquine.
- To provide evidence that the *IDH1/2* mutational status of chondrosarcoma, glioma and intrahepatic cholangiocarcinoma can be assessed with better sensitivity and specificity using enantiomer-specific measurements that determine the separate *D*-

2HG and *L*-2HG levels in serum, urine, and bile than with measurements that determine total 2HG concentrations;

- To provide evidence that the *IDH1/2* mutational status of chondrosarcoma and intrahepatic cholangiocarcinoma patients can be determined by MRS-facilitated detection of intratumoural 2HG levels or liquid biopsies;
- To provide evidence of activity of metformin plus chloroquine related to *D*-2HG levels in the serum, urine, bile, and/or tumoural mass of patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma.

Trial end points

Primary end points

- We will determine the MTD, which is the chloroquine plus metformin dose in which ≤1 in three patients (of a 3+3 dose-escalation schedule) show serious adverse effects.
- We will determine the RD of chloroquine plus metformin, which is the dose level one step below the MTD.

Secondary end points

- Serum metformin and chloroquine concentrations will be measured to investigate the pharmacokinetics of this combination and establish a relationship or not between drug exposure and toxicity and/or efficacy.

- Tumour size will be measured using a MRI and/or CT scan before and after treatment with metformin plus chloroquine to monitor tumour response, using response evaluation criteria in solid tumours (RECIST) 1.1 in chondrosarcoma and intrahepatic cholangiocarcinoma patients and response assessment in neuro-oncology (RANO) in glioma patients.
- *D*-2HG concentrations in serum, urine, bile, and/or the tumoural mass will be measured by MS every four weeks during treatment and by MRS at the start and end of the treatment to investigate the effects of metformin plus chloroquine on *D*-2HG levels. Furthermore, these *D*-2HG measurements will be compared with results obtained from CT and/or MRI scans to investigate whether determinations of *D*-2HG concentrations in serum, urine, bile, and/or the tumoural mass correlate with radiologically observed tumour responses to therapy.
- The variant allelic frequency of *IDH1* mutations or *IDH2* mutations will be measured using NGS on liquid biopsies at the start and end of the treatment and every four weeks during treatment to determine the effects of metformin plus chloroquine on the variant allelic frequency and mutational load of these mutations.

Participants

In brief, this trial will enrol eligible patients with *IDH1/2*-mutated and newly-diagnosed, recurrent and/or metastasized WHO grade II-III chondrosarcoma,[58] WHO grade II-IV glioma,[59] or intrahepatic cholangiocarcinoma. All inclusion and exclusion criteria are listed in **Table 1**. The trial will also enrol patients who have a tumour (re-)resection planned. These patients will be studied in their waiting period until resection (approximately 6-8 weeks). In those cases, the study ends two days before surgery. We are especially interested in patients that had a tumour resection in the past of which tumour material is available, who

had a recurrence of their tumour and who will have a re-resection of this recurrent tumour, because we will then be able to collect pre- and post-treatment samples of these patients. This may also be achieved using sequential tumour biopsies.

This phase Ib/II dose-finding study has three dose-escalation levels. According to a 3+3 dose-escalation scheme, we need a maximum of 18 patients (a maximum of 6 patients in 3 dose escalation levels). A maximum of 10 patients can be enrolled of each tumour type (chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma).

Dose of study drugs and dose escalation schedule

Metformin

The starting dose of metformin will be 500 mg per os q.d. during the first five days. Subsequently, the metformin dose will be escalated as outlined in **Table 2**. This escalation schedule is based on an earlier phase II clinical trial in pancreatic adenocarcinoma.[60] The purpose of the lower metformin starting dose is to reduce side effects of metformin, especially gastro-intestinal side effects. This starting dose mimics dosage schedules of metformin treatment in patients with type 2 diabetes mellitus.

Chloroquine

Chloroquine will be added to metformin in week 2 of the study and chloroquine doses will not be escalated.

- Patients who have no tumour resection planned will be treated with 200 mg chloroquine q.d.
- For patients who have a tumour resection planned, chloroquine will be given in a step-down dosing schedule. The starting dose (first two weeks of chloroquine administration; week 2 and 3 of study) is 300 mg q.d. In subsequent weeks (week 4 of the study and later), the chloroquine maintenance dose will be 200 mg q.d. Because we expect the study duration to be a few weeks in patients with resectable tumours (there usually is a waiting time of 6-8 weeks from diagnosis until surgery), the higher starting dose in patients with resectable tumours allows build-up of functional chloroquine serum concentrations in a shorter time, thereby increasing the chance of a measurable effect within the period of time in which the study will be conducted. This dosing schedule is necessary because of the long half-life of chloroquine. Step-down dosing schedules of chloroquine are also used in systematic lupus erythematodes.[61]

Dose finding

The rate of subject entry and escalation to the next dose level will depend upon assessment of the safety profile of patients entered at the previous dose level. Toxicity will be evaluated according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. A minimum of 3 patients will be entered on each dose level. **Supplementary File 1** describes the standard 3+3 dose-escalation schedule of our study, the procedures for intrapatient dose-escalation and patient replacement and the finding of the recommended phase II dose. Dose (de)escalation will be based on the toxicity assessment in the first eight weeks of therapy and the documentation of any dose-limiting toxicities (DLTs). To be considered as a DLT, the toxicity must be considered to be related to the study drug. DLTs

are defined in **Supplementary Table 1.** When a patient experiences a DLT, he/she can decide to withdraw from the study or go into intrapatient de-escalation by receiving metformin at one dose level lower than the dose level that provoked the DLT.

Study visits

Screening

Patients will visit their hospital of inclusion once or twice for screening purposes, depending on whether or not the *IDH1/2* mutational status is known from DNA sequencing or immunohistochemistry. When there is no *IDH1/2* mutational status or tumour material available, patients will undergo a pre-screening study visit to assess the *IDH1/2* status by drawing blood or collecting urine to perform MS for *D*-2HG levels or by MRS to determine the 2HG level in their tumour.

Specifics for patients who will have no tumour resection

Patients who have no tumour resection planned will undergo a study visit after one week, in which blood will be drawn for pharmacokinetic analysis (see below) and after four weeks, in which blood will be drawn for serum *D*-2HG MS analysis, for analysis of hematologic, hepatic, renal, and chemistry parameters and for further pharmacokinetic analyses. Every eight weeks, these patients will have a more elaborate study visit in which they will undergo a CT/MRI scan in addition to the procedures that will also occur at study visits every four weeks. Specifics for each study visit are shown in **Table 3**. The end of study is defined as

when a patient chooses to withdraw from the study, when a patient experiences a DLT, or when tumour progression occurs.

Specifics for patients who have a tumour resection planned

For patients who will have a tumour resection, the study will be conducted during the waiting period until surgery, and will end two days before surgery. This group also includes patients that already had a tumour resection but will have a re-resection because their tumour recurred. By approval of the patient, the *D*-2HG serum concentration can already be measured using MS during the patient's consideration to participate in the trial or not in order to speed up the screening procedure and prolong the study duration in which the patient can be treated. For this blood drawing and MS analysis of serum, the patient can give informed consent separately, *i.e.* without already having to consent to inclusion in the full clinical trial. Patients will undergo study visits after one and four weeks that are similar to study visits of patients that will have no tumour resection. Specifics for each study visit are shown in **Table 3**. The end of study is defined as when a patient chooses to withdraw from the study, when a patient experiences a DLT, or two days before surgery.

Pharmacokinetics

Pharmacokinetics of metformin and chloroquine are monitored in order to evaluate a relationship between drug exposure, toxicity, and/or efficacy. Furthermore, the magnitude of the pharmacokinetic interactions between both compounds will be assessed. Blood samples will be taken at several time points during the study for the determination of the respective plasma levels.

The half-life of metformin is ±6.5 hours,[62] which means that with daily dosing the plasma level of metformin reaches a steady-state concentration within two days. The half-life of chloroquine is considerably longer (±2 weeks),[63] which means that with daily dosing the plasma level of chloroquine reaches a steady-state concentration within eight weeks in a flat-dosed scheme (which applies to patients who will have no tumour resection) and ±4-6 weeks under the proposed step-down dose scheme (see above).

Predose plasma samples (*i.e.* prior to study medication ingestion) will be taken on day 8 (week 2), day 29 (week 5), every four weeks thereafter and at the end of the study (see **Table 3**). Because chloroquine administration starts on day 8, the predose plasma sample on that day contains a metformin plasma concentration that reflects metformin monotherapy. The pharmacokinetic interaction between metformin and chloroquine is evaluated by comparing the metformin concentration on day 8 with the metformin concentration at subsequent time points. The relationship between exposure and toxicity is evaluated using all samples. The difference in the time after which steady-state serum levels of metformin and chloroquine are reached also help with distinguishing the source of any drug-related toxicity, because any toxicity in the first month is unlikely to be the result of chloroquine, but likely the result of metformin.

Detection of *D*-2HG levels in serum, urine, and/or bile

We will detect *D*-2HG levels in patient serum, urine, and/or bile using MS. Because our method distinguished the *IDH1/2* mutation-specific *D*-2HG from the unspecific *L*-2HG, we expect a better signal-to-noise ratio and a higher sensitivity and specificity to detect *IDH1/2* mutations than in previous studies, where total 2HG levels were measured.[48 49 53] Bile

samples will only be obtained from patients with intrahepatic cholangiocarcinoma with easy access to bile samples in the context of regular patient care, such as a percutaneous transhepatic biliary drain.

Detection of intratumoural 2HG levels

Intratumoural 2HG levels will be detected using long-echo MRS (PRESS) on a 3T MRI at the start and end of treatment of patients using protocols that were described before.[51] We will compare intratumoural 2HG levels before and after treatment to investigate whether MRS can be used to monitor therapy responses in *IDH1/2*-mutated solid tumours. We will also compare results from MRS with the results of DNA sequencing or immunohistochemistry to investigate whether MRS can be used to determine the mutational status of *IDH1/2* in patients with chondrosarcoma or intrahepatic cholangiocarcinoma.

Therapy response assessment

Response will be assessed by RECIST 1.1 guidelines[64] for chondrosarcoma and intrahepatic cholangiocarcinoma or RANO guidelines[65] for glioma on images obtained with CT and/or MRI scans. Scans will be performed at screening and every eight weeks from study inclusion.

ctDNA and plasma fractions, derived from blood samples that will be taken before, during and after the study treatment, will be sequenced using NGS on an IonTorrent platform to determine the *IDH1/2* mutational burden and analysed for *D*-2HG levels using MS to determine the metabolic activity of the *IDH1/2*-mutated tumour, respectively. This will also yield sensitivity and specificity parameters for these investigational diagnostic modalities.

When there is pre-study and post-study primary tumour material available, either obtained during surgery or a tumour biopsy in the context of regular patient care or obtained during a tumour biopsy specifically for this clinical trial, we will perform immunohistochemical staining with the appropriate IDH1/2 mutant-specific antibody to investigate the intratumoural mutational burden.

Ethics and dissemination

An ethical limitation of the present clinical trial may be that the therapeutic index of metformin and chloroquine has been established in glioma and colorectal carcinoma cells,[16] but not in intrahepatic cholangiocarcinoma or chondrosarcoma models. However, this is primarily a dose-finding study. Follow-up phase II clinical trials will be rationally designed based on the pending evidence whether or not the efficacy of metformin and chloroquine treatment will be validated in model systems of other types of cancer by then. Further statements on ethics and dissemination can be found in **Supplementary File 1**.

LIST OF ABBREVIATIONS

αKG	alpha-ketoglutarate
AML	acute myeloid leukaemia
BBB	blood-brain barrier
b.i.d.	bis in die, two times a day
CTCAE	common terminology criteria for adverse events
ctDNA	circulating tumour DNA
D-2HG	*D*-2-hydroxyglutarate
DLT	dose-limiting toxicity
ETC	electron transport chain
IDH1/2	isocitrate dehydrogenase 1 or 2
$IDH1/2^{WT}$	IDH1/2 wild-type
$IDH1/2^{MT}$	IDH1/2 mutant
MAD	maximum administered dose
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
MTD	maximum tolerated dose
NGS	next-generation sequencing

q.d.	quaque die, one a day
RANO	response assessment in neuro-oncology
RD	recommended dose (for a phase II clinical trial)
RECIST	response evaluation criteria in solid tumours
TCA cycle	tricarboxylic acid cycle

DECLARATIONS

Ethics approval and consent to participate

Ethical approval was obtained from the medical-ethical review committee (METC) of the Academic Medical Centre, Amsterdam, the Netherlands and the Dutch national competent authority (CCMO) on 22 October 2015 under reference number NL53150.018.15.

Consent for publication

Not applicable.

Availability of data and material

- Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests:

The authors declare no conflict of interest.

Funding:

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Authors' contributions

R.J.M. and J.W.W. conceived and designed the study. R.J.S., M.Kh., M.E.v.L., M.Ko., J.A.M.B., J.V.M.G.B., R.A.A., H.J.K, H.W.M.v.L., C.J.F.v.N., W.P.V., H.G. and T.M.v.G. guided the study design. R.J.M., M.Kh. and M.W.A.C. performed pilot study experiments. R.J.M. wrote the manuscript. All authors read and approved the final manuscript.

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Registration:

This article was registered at ClinicalTrials.gov under identifier NCT02496741.

Study dates:

Date of study registration: 30 June 2015

Date of ethical approval: 22 October 2015

Date of first enrollment: 17 November 2015

Patients included as of 25 October 2015: 3.

Expected date of enrollment completion: Q4 2017.

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FIGURE LEGENDS

Figure 1. Cellular carbohydrate and glutamine metabolism showing the pathways in which wild-type IDH1 and IDH2 are functional. Isocitrate dehydrogenase 1, 2, and 3 (IDH1/2/3) catalyse the conversion of isocitrate to α-ketoglutarate (αKG) in the cytoplasm and mitochondria, respectively, where IDH1 and IDH2 are NADPH-producing and IDH3 is NADH-producing. This reaction occurs in tricarboxylic acid (TCA) cycle(-like) metabolism and is fed by glucose influx and/or glutamine influx. The conversion of glutamine into αKG occurs in the glutaminolysis pathway and the last step is catalysed by glutamate dehydrogenase (GDH), which is inhibited by chloroquine and metformin. The TCA cycle produces NADH, which is used in the electron transport chain (ETC) and oxidative phosphorylation to generate ATP. Complex I of the ETC is inhibited by metformin. Wild-type IDH1/2 ($IDH1/2^{WT}$) differs from mutant IDH1/2 ($IDH1/2^{MUT}$) because the latter enzyme converts αKG into a novel oncometabolite, *D*-2-hydroxyglutarate (*D*-2HG).

Figure 2. Dosing schedule and study design for patients who will not undergo tumour resection. Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

Figure 3. Dosing schedule and study design for patients who have a tumour resection planned. Surgery will define the end of this time schedule (2 days before surgery). Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

TABLES AND LEGENDS

Table 1. Inclusion and exclusion criteria.

Key inclusion criteria
• Age ≥18 years. • Presence of a measurable intrahepatic cholangiocarcinoma or WHO grade II-III chondrosarcoma (RECIST 1.1 criteria [64]) or WHO grade II-IV glioma (RANO criteria [65]), both newly-diagnosed and refractory/relapsed tumours. • Tumour carries a *D*-2HG-generating mutation in *IDH1* or *IDH2* as determined by sequencing of primary tumour DNA or ctDNA, immunohistochemistry of primary tumour tissue with an IDH1/2 mutant-specific antibody, MS of serum, urine, and/or bile or MRS imaging of the tumour. • ECOG/WHO performance 0-2. • Adequate renal function (creatinine <150 µmol/L or a creatinine clearance >60 ml/L). • Adequate liver function (bilirubin <1.5 times the normal upper limit; ALAT and ASAT <2.5 the normal upper limit). • Adequate bone marrow function (white blood cells >3.0 x 10^9/L, platelets >100 x 10^9/L). • When patient is eligible for tumour resection, surgery is (already) planned at least 4 weeks later than the start of study treatment.
Key exclusion criteria

• Concomitant other anti-cancer therapy (i.e. surgical resection, chemotherapy, targeted therapy, radiation therapy, surgery). Palliative therapy is permitted, such as:
  ○ palliative radiotherapy for symptomatic bone metastases,
  ○ dexamethasone for symptom relief in patients with glioma and cerebral oedema,

- non-enzyme inducing anti-epileptic drugs (with the exception of topiramate) in patients with glioma and epileptic seizures.

- Severe and/or uncontrolled medical conditions at <6 months prior to randomization, such as:
  - unstable angina pectoris, symptomatic congestive heart failure, myocardial infarction or cardiac arrhythmias,
  - pulmonary insufficiency,
  - epilepsy (interaction with chloroquine),
  - severe gastrointestinal, neurological or hematological diseases (interaction with chloroquine),
  - uncontrolled diabetes as defined by fasting serum glucose >12 mmol/l,
  - active or uncontrolled severe infection, including malaria,
  - cirrhosis, chronic active hepatitis or chronic persistent hepatitis.
- Serious concomitant systemic disorder that compromises the safety of the patient, at the discretion of the investigator.
- Patients who have a known history of alcohol abuse (interaction with metformin).
- Patients with known glucose-6-phosphate dehydrogenase deficiency, porphyria, myasthenia gravis or ocular/retinal aberrations (interactions with chloroquine).
- Patients who use digoxin, MAO inhibitors, fenylbutazone, oxygenbutazone, gold preparations or cimetidine (known pharmacokinetic interactions with chloroquine) or loop diuretics (known pharmacokinetic interaction with metformin) for which not a good alternative is available.
- Patients with a known hypersensitivity to metformin or chloroquine.
- Use of metformin or chloroquine in the previous 6 months or long-term use of chloroquine (>5 years or cumulative dose >300 grams) in the past.

Abbreviations: ctDNA, circulating tumour DNA; *D*-2HG, *D*-2-hydroxyglutarate; IDH1/2, isocitrate dehydrogenase 1 and 2; MRS, magnetic resonance spectroscopy; MS, mass spectrometry.

Table 2. Metformin dose escalation schedule.

Dose level	Dose of metformin given orally (total daily dose)	Minimum number of patients
-1	500 mg q.d. (500 mg total)	--
1 (starting)	500 mg b.i.d. (1000 mg total)	3
2	1000 mg b.i.d. (2000 mg total)	3
3	1500 mg b.i.d. (3000 mg total)	3

Abbreviations: b.i.d., two times a day; q.d., once a day.

Table 3. Timeline, study treatment, study visits and medical procedures.

Required investigations	Screening	Day 8 (week 2)	Day 29/week 5 and every 4 weeks thereafter	Day 57/week 9 and every 8 weeks thereafter	End of study
Visit number	1	2	3	4+	4+
Written informed consent	Prior to Screening				
Demographics (age, sex)	X				
Overall medical	X				X

history					
Physical examination, including weight and height	X		X	X	X
Vital signs (blood pressure, pulse)	X		X	X	X
ECOG/WHO performance status	X		X	X	X
CT or MRI scan of measurable lesion, ≤1 month prior to start treatment	X			X	X
Haematology	X		X	X	X
Serum chemistry:					
Hepatic function	X		X	X	X
Renal function	X		X	X	X
Glucose	X		X	X	X
HbA1c	X			X	X
Triglycerides	X			X	X
Cholesterol	X			X	X
Haemostatic parameters (aPPT and PT)	X				X
Insulin, IGF-1,	X			X	X

IGF binding protein-3					
Metformin concentration		X	X	X	X
Chloroquine concentration			X	X	X
MS of serum/urine/bile for *D*-2HG levels	X		X	X	X
MRS for intratumoural 2HG levels	X				X
Liquid biopsy	X		X	X	X
ECG	X				
Pregnancy test	X				
Optional: tumour biopsy	X			X	X

In addition to this scheme, an ECG will be performed every 24 weeks. Abbreviations: (*D*-)2HG, (*D*-)2-hydroxyglutarate; IGF, insulin growth factor; MRS, magnetic resonance spectroscopy; MS, mass spectroscopy.

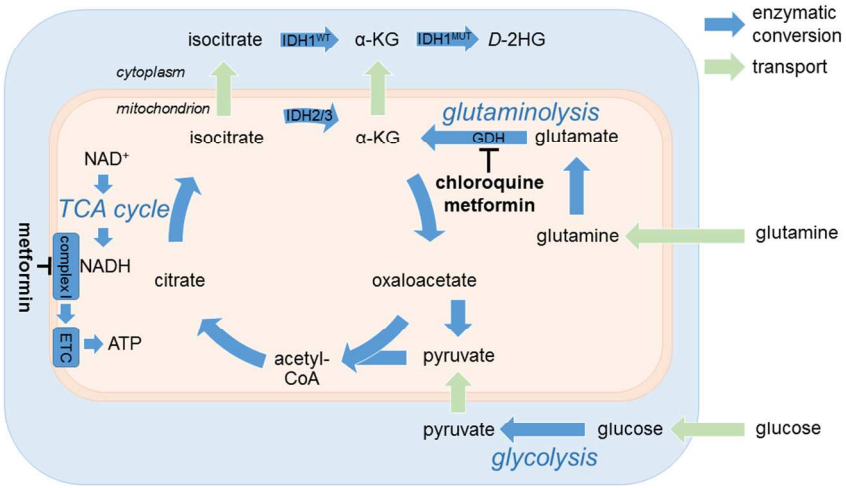

Figure 1. Cellular carbohydrate and glutamine metabolism showing the pathways in which wild-type IDH1 and IDH2 are functional. Isocitrate dehydrogenase 1, 2, and 3 (IDH1/2/3) catalyse the conversion of isocitrate to α-ketoglutarate (αKG) in the cytoplasm and mitochondria, respectively, where IDH1 and IDH2 are NADPH-producing and IDH3 is NADH-producing. This reaction occurs in tricarboxylic acid (TCA) cycle(-like) metabolism and is fed by glucose influx and/or glutamine influx. The conversion of glutamine into αKG occurs in the glutaminolysis pathway and the last step is catalysed by glutamate dehydrogenase (GDH), which is inhibited by chloroquine and metformin. The TCA cycle produces NADH, which is used in the electron transport chain (ETC) and oxidative phosphorylation to generate ATP. Complex I of the ETC is inhibited by metformin. Wild-type IDH1/2 (IDH1/2^{WT}WT) differs from mutant IDH1/2 (IDH1/2^{MUT}) because the latter enzyme converts αKG into a novel oncometabolite, *D*-2-hydroxyglutarate (*D*-2HG).

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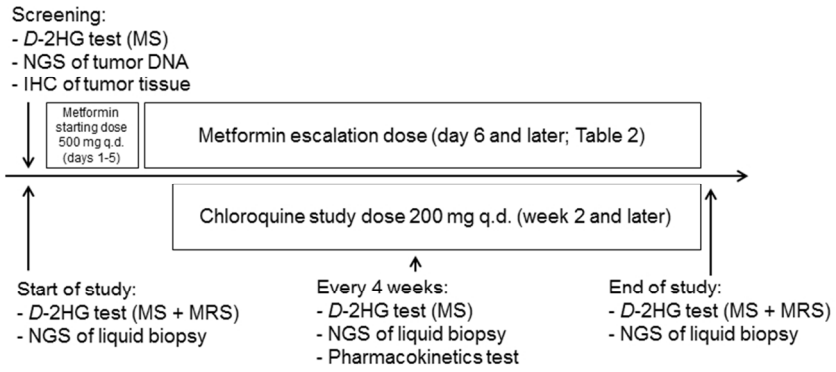

Figure 2. Dosing schedule and study design for patients who will not undergo tumour resection. Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

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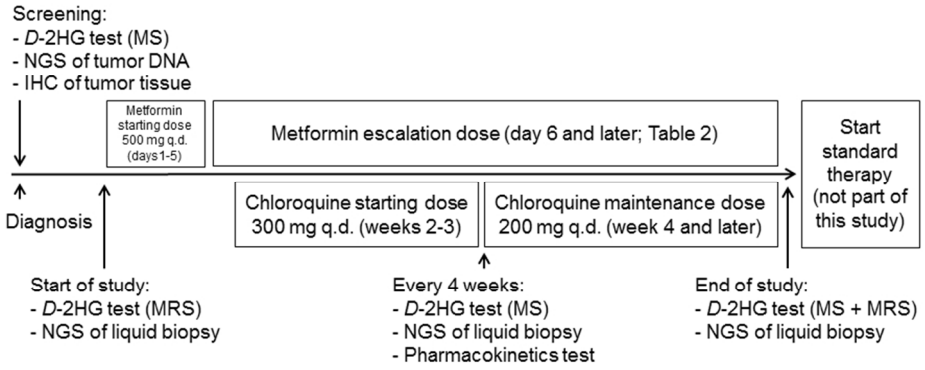

Figure 3. Dosing schedule and study design for patients who have a tumour resection planned. Surgery will define the end of this time schedule (2 days before surgery). Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

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SUPPLEMENTARY FILE 1

3+3 dose-escalation schedule and intrapatient dose-escalation

Maximum administered dose

If 0/3 patients exhibit dose-limiting toxicity at this dose level:

- Dose escalation to the next dose level may begin in a new cohort of patients
- Patients enrolled on the previous dose level who are still receiving therapy may now undergo intrapatient dose escalation to this new dose level provided that they have experienced no drug related toxicity of grade 2 or higher at the previous dose level.

If 1/3 patients exhibit dose-limiting toxicity at this dose level:

- Expand dose level to a total of six patients. Toxicity information from patients who underwent intrapatient dose escalation can be used for expansion cohorts, but only when they have completed at least 8 weeks of treatment at the new dose level.
- If no further DLT events are observed, dose escalation to the next dose level may begin in a new cohort of patients and patients enrolled on the previous dose level who are still receiving therapy may now undergo intrapatient dose escalation to this dose level provided that they have experienced no drug related toxicity of grade 2 or higher.
- If further DLTs are observed (i.e. in ≥2/6 patients), this dose level will be considered the maximum administered dose (MAD).

If ≥2/3 patients exhibit dose-limiting toxicity

- This dose level will be considered the MAD.
- If this toxicity occurs at level 1 (starting level), dose de-escalation to level -1 will be applied.

Recommended phase II dose

As described in the full text manuscript, the MAD is the dose in which ≥2/3 or ≥2/6 patients experience a DLT, or the final dose from the dose escalation schedule (1500 mg metformin b.i.d. and 200 mg chloroquine q.d.). One dose level below the MAD will be considered the RD for follow-up phase II clinical trials. When the starting dose level (“1”) is the MAD, we will de-escalate the dose level to dose level “-1”. When we do not observe DLTs in three patients or one DLT in six patients at this dose level, then dose level “-1” will be the RD. When we observe more than one DLT, the combination of metformin and chloroquine will be considered too toxic to be useful in cancer patients. In contrast to this situation where we have to accept the lowest dose-escalation level as the RD, when 0/6 patients experience DLTs at the final dose level of the dose escalation schedule (i.e. dose level “3”), this can be considered the RD for follow-up phase II clinical trials, instead of dose level “2”.

Up to a total of six patients may be treated at the RD level to assure information on the safety profile when that dose is complete. When clinically appropriate, intermediate dose levels may be studied to assure that the RD is the highest tolerable. Furthermore, when pharmacokinetic data suggests that saturating absorption of drug is occurring on a b.i.d. oral administration

level, further dose splitting to three times a day or four times a day schedules may be considered.

Patient replacement

Three patients within a dose level must be observed for eight weeks before accrual to the next dose level may begin. If a patient is withdrawn from the study prior to completing 22 days of therapy without experiencing a DLT prior to withdrawal, an additional patient may be added to that dose level. Patients missing seven or more doses (one week) due to toxicity will not be replaced since these patients will be considered to have experienced a dose-limiting toxicity.

Ethics and dissemination

This study is being conducted according to Good Clinical Practice guidelines as described in the International Conference on Harmonization Guideline E6 and in accordance with general ethical principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the medical-ethical review committee (METC) of the Academic Medical Centre, Amsterdam, the Netherlands and the Dutch national competent authority (CCMO) on 22 October 2015 under reference number NL53150.018.15.

A report describing the results of the study will be submitted to a peer-reviewed journal. Where permitted by patient data protection standards, data will be published and shared together with the publication of the study results.

Supplementary Table 1. Hematologic and non-hematologic dose-limiting toxicities.

Hematologic	Non-hematologic
• Absolute granulocyte count <0.5 x 10^9/l. • Febrile neutropenia (ANC <1.0 x 10^9/L, fever >38.5°C). • Platelets <25 x 10^9/l. • Bleeding due to thrombocytopenia, as determined by a physician.	• Diarrhoea > grade 3 despite optimal loperamide use. • Rash > grade 3 or grade 2 is medically concerning or unacceptable to the patient. • Other grade 3 effects considered to be treatment related. • Missing >7 days of treatment for toxicity reasons.

Grading of side effects is performed using CTCAE. Abbreviations: ANC, absolute neutrophil count.

BMJ Open

Study protocol of a phase Ib/II clinical trial of metformin and chloroquine in patients with *IDH1*-mutated or *IDH2*-mutated solid tumors

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STUDY PROTOCOL OF A PHASE IB/II CLINICAL TRIAL OF METFORMIN AND CHLOROQUINE IN PATIENTS WITH *IDH1*-MUTATED OR *IDH2*-MUTATED SOLID TUMOURS

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ABSTRACT

Introduction: High-grade chondrosarcoma, high-grade glioma, and intrahepatic cholangiocarcinoma are aggressive types of cancer with a dismal outcome. This is due to the lack of effective treatment options, emphasizing the need for novel therapies. Mutations in the genes *IDH1* and *IDH2* occur in 60% of chondrosarcoma, 80% of WHO grade II-IV glioma and 20% of intrahepatic cholangiocarcinoma. *IDH1/2*-mutated cancer cells produce the oncometabolite *D*-2-hydroxyglutarate (*D*-2HG) and are metabolically vulnerable to treatment with the oral antidiabetic metformin and the oral antimalarial drug chloroquine.

Methods and analysis: We describe a dose-finding phase Ib/II clinical trial, in which patients with *IDH1/2*-mutated chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma are treated with a combination of metformin and chloroquine. Dose escalation is performed according to a 3+3 dose-escalation scheme. The primary objective is to determine the maximum tolerated dose to establish the recommended dose for a phase II clinical trial. Secondary objectives of the study include (1) determination of pharmacokinetics and toxic effects of the study therapy, for which metformin and chloroquine serum levels will be determined over time; (2) investigation of tumour responses to metformin plus chloroquine in *IDH1/2*-mutated cancers using CT/MRI scans; and (3) whether or not tumour responses can be measured by non-invasive *D*-2HG measurements (mass spectrometry (MS) and magnetic resonance spectroscopy (MRS)) of tumour tissue, serum, urine, and/or bile or next-generation sequencing of circulating tumour DNA (liquid biopsies). This study may open a novel treatment avenue for *IDH1/2*-mutated high-grade chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma by repurposing the combination of two inexpensive drugs that are already approved for other indications.

Ethics and dissemination: This study has been approved by the medical-ethical review committee of the Academic Medical Center, Amsterdam, The Netherlands. The report will be submitted to a peer-reviewed journal.

STRENGTHS AND LIMITATIONS OF THIS STUDY

Strengths:

- Metformin and chloroquine are two safe, inexpensive drugs that are already approved for other indications.
- Whereas the dependence of late-stage malignant tumours on individual oncogenic mutations decreases, the metabolic stress that results from *IDH1/2* mutations persists. Therefore, metabolic drugs such as metformin and chloroquine may carry favourable therapeutic efficacies irrespective of the tumour's stage.

Limitations:

- Because this is primarily a dose-finding study, we may not be able to study the efficacy of metformin and chloroquine.
- When patients do not consent to tumour biopsies/re-resections, this diminishes the possibility for translational analyses.

ARTICLE MANUSCRIPT

Introduction

IDH1 and IDH2 are homodimeric enzymes that reversibly convert isocitrate to α-ketoglutarate (αKG) in cytoplasm and mitochondria, respectively. Somatic heterozygous mutations in *IDH1/2* that produce the oncometabolite *D*-2-hydroxyglutarate (*D*-2HG) are observed in substantial percentages of various tumour types such as chondrosarcoma (60%), WHO grade II-III glioma (80%), secondary WHO grade IV glioblastoma (80%), and intrahepatic cholangiocarcinoma (20%).[1] In addition, *IDH1/2* mutations occur in varying percentages of acute lymphocytic leukaemia (10%), acute myeloid leukaemia (AML; 20%), angioimmunoblastic T-cell lymphoma (40%), colorectal cancer (5%), and melanoma (12%).[1] In chondrosarcoma and glioma, *IDH1/2* mutations are considered very early or even inaugural genetic defects, and are thus present in a large fraction of, or even all, cancer cells.[2 3] This renders *IDH1/2* mutations an interesting target for anti-cancer treatment because such tumour homogeneity decreases the risk of therapy resistance.[4] Recently, inhibitors of mutant IDH1 and IDH2 were developed that may be effective in stalling malignant progression of early-stage *IDH1/2*-mutated cancers.[5 6]

Prognosis and therapeutic options of cancers in which *IDH1/2* mutations occur

The prognosis of solid tumours with frequent occurrence of *IDH1/2* mutations remains poor. The current standard therapy for chrondrosarcoma is surgery. There is no evidence for a benefit of (adjuvant) radiotherapy or chemotherapy, as chondrosarcoma are considered to be highly therapy resistant.[7] Consequently, the 1-year survival rate of metastasized high-grade chondrosarcoma is <10%.[8]Gliomas vary from WHO grade II diffuse astrocytoma and diffuse oligodendroglioma, with median survivals of more than five years,[9] to WHO grade IV glioblastoma, with a median survival of only 15 months despite aggressive treatment using

radiotherapy and temozolomide.[10] Gliomas are diffusely growing tumours, which renders surgery ineffective, emphasizing the dire need for novel therapies. Furthermore, the blood-brain barrier (BBB) prohibits the use of most chemotherapeutics and the surrounding normal brain hampers aggressive radiotherapy regimens due to limitations that are raised by healthy brain tissue.[11] Intrahepatic cholangiocarcinoma is resectable in only 40% of patients.[12] In unresectable cases, intrahepatic cholangiocarcinoma patients are offered palliative treatment as standard of care with the chemotherapy combination of cisplatin and gemcitabine, with a median overall survival of 11.7 months.[13]

Metabolic effects of *IDH1/2* mutations

Heterozygous hotspot *IDH1/2* mutations disable IDH1/2 wild-type enzyme activity[14-16] and induce a neo-enzymatic activity that leads to the production and subsequent accumulation of *D*-2HG.[17-19] *D*-2HG is normally present only in trace amounts in normal tissues and cells but accumulates up to 50 mM in *IDH1/2*-mutated glioma.[17] *D*-2HG is chemically very similar to α-ketoglutarate (αKG) and inhibits over 60 αKG-dependent enzymes, resulting in global DNA/histone hypermethylation, decreased hypoxia-inducible factor 1a (HIF1α) expression, and perturbed collagen maturation.[1] Depending on the cellular context, these effects are the basis of oncogenesis and imply a dependence on *D*-2HG of early-stage *IDH1/2*-mutated tumours.[1]

IDH1/2-mutated cancer cells need αKG to synthesize *D*-2HG and fuel the tricarboxylic acid (TCA) cycle to support their metabolism. αKG is generated by glycolysis (glucose breakdown) or glutaminolysis (glutamine/glutamate breakdown).[20] *IDH1/2* mutations

downregulate αKG levels by consuming αKG and by inhibition of αKG production via direct effects, *i.e.* by disabling *IDH1/2* wild-type kinetics, and indirect effects, *e.g.* by decreasing TCA cycle activity.[1] Therefore, *IDH1/2*-mutated cancer cells rely on glutaminolysis for sufficient αKG supply to generate the oncometabolite *D*-2HG (**Figure 1**).[21] The conversion of glutamate to αKG is catalysed by glutamate dehydrogenase (GDH), which is the final step of glutaminolysis and can be inhibited by the anti-malaria drug chloroquine and the antidiabetic drug metformin.[20 22-24] In addition, *IDH1*-mutated glioma cells show increased levels of autophagy, likely as a survival mechanism of cells to metabolic stress by catabolizing proteins in order to provide substrates for energy production in stress/starvation contexts.[25] Autophagy is inhibited by chloroquine[26] and the anti-cancer properties of chloroquine may thus be selective for *IDH1/2*-mutated cells because it inhibits glutaminolysis and autophagy on which the cells are dependent.

IDH1/2 mutations induce further metabolic stress in *IDH1/2*-mutated cancer cells via inhibition of the TCA cycle and electron transport chain (ETC) by *D*-2HG. More specifically, *D*-2HG inhibits enzymatic activity of complex IV (cytochrome C oxidase) of the ETC[27] and the TCA(-like) enzymes IDH1/2 and αKG dehydrogenase.[16] This reduces oxidative phosphorylation, the primary source of ATP in cancer cells.[27 28] This metabolic stress is amplified *in vitro* in *IDH1*-mutated glioma and colorectal carcinoma cells using compounds that inhibit ETC complex I, such as the oral antidiabetic biguanide metformin and phenformin, which selectively restrict the proliferation of these cells.[16 28]

Another metabolic vulnerability of *IDH1/2*-mutated glioma may be their excess deposition of the acidic *D*-2HG in their microenvironment, which is hypothesized to contribute to their diffuse growth.[29 30] Chloroquine buffers the tumor[31] and may decrease this acidification, reduce the diffuse growth and ultimately increase the treatability of *IDH1/2*-mutated glioma.

Metabolism of *IDH1/2*-mutated tumours as therapeutic target

Metformin and chloroquine increase metabolic stress in *IDH1/2*-mutated cells, as is described above. Patients with *IDH1/2*-mutated glioblastoma have a prolonged survival and better radiotherapy/chemotherapy response when compared with *IDH1/2* wild-type counterparts,[14 32 33] while in chondrosarcoma a correlation between mutation and survival was absent.[2] We and others have shown that *IDH1/2* mutations sensitize glioma and colorectal carcinoma cells to therapies that involve oxidative stress, such as radiotherapy, cisplatin, and carmustine.[16 34 35] Combined, these data suggest that at least some types of cancer with *IDH1/2* mutations should be targeted by compounds that exploit this presumed metabolic vulnerability rather than compounds that decrease metabolic stress (*i.e.* IDH1/2-mutant inhibitors). Accordingly, we hypothesized that the difference in survival of patients with *IDH1/2*-mutated glioma or intrahepatic cholangiocarcinoma versus *IDH1/2* wild-type counterparts is caused by dysregulation of cellular defence mechanisms by *IDH1/2* mutations against anti-cancer therapy.[1 16 36] Little is known about the role of *IDH1/2* mutations in late-stage cancer. It is plausible that with increasing mutational burden, the dependence of late-stage malignant tumours on *IDH1/2* mutations decreases, diminishing the therapeutic index of IDH1/2-mutant inhibitors.[37 38] On the other hand, metabolic stress that results from *IDH1/2* mutations persists, and this metabolic vulnerability provides an excellent target for therapy irrespective of the tumour stage.

Discussion regarding the use of metformin and chloroquine

Metformin and chloroquine are readily-available, inexpensive, and safe drugs that are already FDA/EMA-approved for other indications. The safety profiles of metformin and

chloroquine are favourable over other anti-cancer modalities, which may aid rapid implementation of these drugs into therapies for patients with *IDH1/2*-mutated cancers. A caveat is that the combined safety of metformin and chloroquine is to be proven by our study, although there are no reports of toxic side-effects of this combination in the literature whereas the prevalence of both diabetes and malaria is high. Since both drugs are off patent, combination treatment with metformin and chloroquine can become a therapeutic advance for patients with *IDH1/2*-mutated solid tumours that is considerably less expensive than products of other anti-cancer research efforts. The potential of metformin and chloroquine as adjuvant drugs was recently demonstrated *in vivo,* where metformin or chloroquine had a sensitizing and/or synergistic anti-tumour effect in combination with temozolomide,[39 40] cisplatin,[41 42] and gemcitabine[43 44] in xenograft models or proof-of-concept clinical trials of various types of human cancer, including glioma. Metformin, but not chloroquine, sensitized xenograft models of various types human cancer to ionizing radiation.[45 46]

Possible concerns may be related to the bioavailability of metformin. We have observed high expression of metformin transporters in chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma cell lines and primary tissue (OCT1-3; The Cancer Cell Line Encylopedia[47] and our own unpublished data). Therefore, we expect to achieve sufficient intratumoural metformin concentrations with dose levels 2 and 3 of our dose-escalation protocol. Whereas millimolar metformin concentrations are necessary to activate the necessary antineoplastic cellular targets *in vitro,* these targets were already activated at ±300-fold lower metformin concentrations *in vivo*. When metformin fails to show any metabolic or anti-tumour effect, we may investigate the feasibility of phenformin treatment in future studies. Phenformin is the lipophilic analogue of metformin which does not depend on transporters to enter cells. However, phenformin has a less favourable safety compared with metformin because it carries an increased risk of inducing lactic acidosis. As a consequence,

phenformin approval for the treatment of diabetes mellitus type 2 was withdrawn by the FDA and EMA in the 1970s[48] and in contrast to metformin, phenformin is not readily available.

With respect to chloroquine, possible concerns may be related to the plethora of cellular targets of chloroquine. Inhibition of autophagy and glutaminolysis and buffering of the tumour milieu are the potential therapeutic targets of chloroquine in *IDH1/2*-mutated cancers. Besides these, chloroquine also induces apoptosis and affects the body's immune response to the tumour *in vitro* and/or *in vivo* at concentrations that may be achieved using the dose that we use in the present clinical trial.[31] These properties of chloroquine as a "dirty drug" may lead to toxicity problems.

For the treatment of glioma, adequate drug penetration of the BBB is necessary for relevant tumour responses. Notwithstanding that high-grade glioma often destruct the BBB, *in vivo* experiments in mice have shown that metformin and chloroquine adequately pass the blood-brain barrier.[49 50]

Non-invasive detection of *IDH1/2* mutations

The gold standard of *IDH1/2* mutation detection is genetic analysis of tumour DNA. In glioma, 90% of all *IDH1/2* mutations are $IDH1^{R132H}$ and its presence can be reliably detected using a immunohistochemistry of glioma tissue with an $IDH1^{R132H}$-specific antibody.[51] The presence of *IDH1/2* mutations in AML[52] and intrahepatic cholangiocarcinoma[53] can be easily, reliably, and non-invasively detected via determination of 2HG levels or *D*-2HG levels in serum or urine by mass spectrometry (MS). Furthermore, MS-determined 2HG serum levels correlate with therapy response in these cancers.[52 53] In a previous study investigating intrahepatic

cholangiocarcinoma, total 2HG levels in serum predicted the presence of an *IDH1/2* mutation (as determined using targeted DNA sequencing) with a sensitivity of 83% and a specificity of 90%.[53]

Whereas no non-invasive detection methods of *IDH1/2* mutations have been described to be effective in chondrosarcoma yet, the presence of *IDH1/2* mutations in glioma can be determined using magnetic resonance spectroscopy (MRS) of the brain, which detects intratumoural 2HG levels.[54 55] Conversely, serum 2HG levels correlate poorly with the *IDH1/2* mutational status in glioma due to a limited blood-brain barrier passage of *D*-2HG.[56] Urine 2HG levels are higher in patients with *IDH1*-mutated glioma than in patients with *IDH1* wild-type glioma,[57] although another study reported decreased 2HG levels in the urine of patients with *IDH1*-mutated glioma and showed that the ratio of serum 2HG levels to urine 2HG levels is most predictive for the *IDH1* mutational status in glioma.[58] Most aforementioned measurements determined total 2HG levels and thus did not discriminate between the *D*-enantiomer of 2HG (which is specific for *IDH1/2* mutations) and the *L*-enantiomer of 2HG (which is unspecific and is generated during hypoxia).[59 60] Better separation of *D*-2HG and *L*-2HG may allow for *IDH1/2* mutational status predictions with higher sensitivity and specificity.

Besides methods that detect *D*-2HG accumulation, *IDH1/2* mutations may also be detected via next-generation sequencing (NGS) of circulating tumour DNA (ctDNA) that is isolated from serum as liquid biopsies. Liquid biopsies contain a collection of ctDNA sequences which is representative for the heterogeneity of the tumour. Therefore, liquid biopsies are more informative than tissue biopsies, which are subject to selection bias as a result of the tumour heterogeneity. In liquid biopsies, variant allelic frequencies can be used as biomarkers for tumour load and dynamic clonal hierarchies within the tumour.[61]

Hypothesis and outlook

To summarize, fundamental and translational research by us and others revealed that *IDH1/2* mutations impart therapeutically targetable metabolic vulnerabilities to cells from several types of cancer.[16 20 21 27 28] We aim to use these metabolic alterations in *IDH1/2*-mutated tumours for screening purposes and tumour response monitoring purposes using non-invasive modalities. Furthermore, we aim to specifically inhibit the metabolic processes that are essential to *IDH1/2*-mutated tumours using metformin and chloroquine, which specifically target the metabolic vulnerabilities that are caused by *IDH1/2* mutations.

We hypothesize that metformin and chloroquine can be safely used as anti-cancer drugs for patients with *IDH1/2*-mutated chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma and that tumour response to treatment can be monitored by measuring tumour size and/or levels of *D*-2HG in serum, urine, bile, and/or the tumoural mass. This hypothesis will be tested in a phase Ib/II clinical trial. There are no reports of clinical trials of combined treatment with metformin and chloroquine yet. In the future, metformin and chloroquine may be used as stand-alone therapy for patients with *IDH1/2*-mutated cancers, especially in chondrosarcoma for which no effective therapies beside surgery exists, or besides conventional anti-cancer treatments such as radiation and temozolomide in glioma and cisplatin and gemcitabine in intrahepatic cholangiocarcinoma.

Methods and analysis

Overall study design

MACIST is a nonrandomized, open-label, dose-finding, multi-centre phase Ib/II clinical trial with a combined regimen of metformin and chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma. Drug dosing will follow a 3+3 dose escalation scheme. Patients will be enrolled at three sites in The Netherlands.

Objectives

Primary objective

To determine the maximum tolerated dose (MTD) and recommended dose (RD) of metformin plus chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma.

Secondary objectives

- To describe the toxic effects and pharmacokinetics of metformin plus chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma;
- To provide evidence of complete or partial tumour regression in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma after treatment with metformin plus chloroquine;
- To provide evidence that the *IDH1/2* mutational status of chondrosarcoma, glioma and intrahepatic cholangiocarcinoma can be assessed using enantiomer-specific measurements that determine the separate *D*-2HG and *L*-2HG levels in serum, urine, or bile (with better sensitivity and specificity than with measurements that determine total 2HG concentrations);

- To provide evidence that the *IDH1/2* mutational status of chondrosarcoma and intrahepatic cholangiocarcinoma patients can be determined by MRS-facilitated detection of intratumoural 2HG levels or liquid biopsies;
- To provide evidence of activity of metformin plus chloroquine related to *D*-2HG levels in the serum, urine, bile, and/or tumoural mass of patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma.

Trial end points

Primary end points

- We will determine the MTD, which is the chloroquine plus metformin dose in which ≤1 in three patients (of a 3+3 dose-escalation schedule) show serious adverse effects.
- We will determine the RD of chloroquine plus metformin, which is the dose level one step below the MTD.

Secondary end points

- Serum metformin and chloroquine concentrations will be measured to investigate the pharmacokinetics of this combination and establish a relationship or not between drug exposure and toxicity and/or efficacy.
- Tumour size will be measured using a MRI and/or CT scan before and after treatment with metformin plus chloroquine to monitor tumour response, using response evaluation criteria in solid tumours (RECIST) 1.1 in chondrosarcoma and intrahepatic

cholangiocarcinoma patients and response assessment in neuro-oncology (RANO) in glioma patients.

- *D*-2HG concentrations in serum, urine, bile, and/or the tumoural mass will be measured by MS every four weeks during treatment and by MRS at the start and end of the treatment to investigate the effects of metformin plus chloroquine on *D*-2HG levels. Furthermore, these *D*-2HG measurements will be compared with results obtained from CT and/or MRI scans to investigate whether determinations of *D*-2HG concentrations in serum, urine, bile, and/or the tumoural mass correlate with radiologically observed tumour responses to therapy.
- The variant allelic frequency of *IDH1* mutations or *IDH2* mutations will be measured using NGS on liquid biopsies at the start and end of the treatment and every four weeks during treatment to determine the effects of metformin plus chloroquine on the variant allelic frequency and mutational load of these mutations.

Participants

In brief, this trial will enrol eligible patients with *IDH1/2*-mutated and newly-diagnosed, recurrent, relapsed or refractory and/or metastasized WHO grade II-III chondrosarcoma,[62] WHO grade II-IV glioma,[63] or intrahepatic cholangiocarcinoma. All inclusion and exclusion criteria are listed in **Table 1**. The trial will enrol patients who have no tumour resection planned (**Figure 2**) and those who have a tumour (re-)resection planned (**Figure 3**). These patients will be studied in their waiting period until resection (approximately 6-8 weeks). We are especially interested in patients that had a tumour resection in the past of which tumour material is available, who had a recurrence of their tumour and who will have a re-resection of this recurrent tumour, because we will then be able to collect pre- and post-treatment samples of these patients. This may also be achieved using sequential tumour biopsies. For

patients that have no tumour resection planned, the end of the study is defined as when a patient chooses to withdraw from the study, when a patient experiences a DLT, or when tumour progression occurs. For patients who will have a tumour resection, the study will be conducted during the waiting period until surgery. The end of study is defined similarly as for patients that have no tumour resection planned or two days before surgery.

This phase Ib/II dose-finding study has three dose-escalation levels. According to a 3+3 dose-escalation scheme, we need a maximum of 18 patients (a maximum of 6 patients in 3 dose escalation levels). A maximum of 10 patients can be enrolled of each tumour type (chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma).

Dose of study drugs and dose escalation schedule

Metformin

The starting dose of metformin will be 500 mg per os q.d. during the first five days. Subsequently, the metformin dose will be escalated as outlined in **Table 2**. This escalation schedule is based on an earlier phase II clinical trial in pancreatic adenocarcinoma.[64] The purpose of the lower metformin starting dose is to reduce side effects of metformin, especially gastro-intestinal side effects. This starting dose mimics dosage schedules of metformin treatment in patients with type 2 diabetes mellitus.

Chloroquine

Chloroquine will be added to metformin in week 2 of the study and chloroquine doses will not be escalated. Patients who have no tumour resection planned will be treated with 200 mg chloroquine q.d. For patients who have a tumour resection planned, chloroquine will be given

in a step-down dosing schedule. The starting dose (first two weeks of chloroquine administration; week 2 and 3 of study) is 300 mg q.d. In subsequent weeks (week 4 of the study and later), the chloroquine maintenance dose will be 200 mg q.d. Because we expect the study duration to be a few weeks in patients with resectable tumours (there usually is a waiting time of 6-8 weeks from diagnosis until surgery), the higher starting dose in patients with resectable tumours allows build-up of functional chloroquine serum concentrations in a shorter time, thereby increasing the chance of a measurable effect within the period of time in which the study will be conducted. This dosing schedule is necessary because of the long half-life of chloroquine. Step-down dosing schedules of chloroquine are also used in systematic lupus erythematodes.[65]

Dose finding

The rate of subject entry and escalation to the next dose level will depend upon assessment of the safety profile of patients entered at the previous dose level. Toxicity will be evaluated according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. A minimum of 3 patients will be entered on each dose level. **Supplementary File 1** describes the standard 3+3 dose-escalation schedule of our study, the procedures for intrapatient dose-escalation and patient replacement and the finding of the recommended phase II dose. Dose (de)escalation will be based on the toxicity assessment in the first eight weeks of therapy and the documentation of any dose-limiting toxicities (DLTs). To be considered as a DLT, the toxicity must be considered to be related to the study drug. DLTs are defined in **Supplementary Table 1.** When a patient experiences a DLT, he/she can decide to withdraw from the study or go into intrapatient de-escalation by receiving metformin at one dose level lower than the dose level that provoked the DLT.

Screening for *IDH1/2* mutations

The *IDH1/2* mutational status of patients will be assessed using DNA sequencing or immunohistochemistry. In a patient with glioma, the presence of an *IDH1/2* mutation can also be established using MRS to detect intratumoural 2HG levels.[54 55]

Study visits

Patients with *IDH1/2*-mutated tumours will visit their hospital of inclusion once for additional eligibility screening (see in- and exclusion criteria). Once enrolled in the study, patients will undergo a study visit after one week, in which blood will be drawn for pharmacokinetic analysis (see below) and after four weeks, in which blood will be drawn for serum *D*-2HG MS analysis, for analysis of hematologic, hepatic, renal, and chemistry parameters and for further pharmacokinetic analyses. Every eight weeks, these patients will have a more elaborate study visit in which they will undergo a CT/MRI scan in addition to the procedures that will also occur at study visits every four weeks. Specifics for each study visit are shown in **Table 3**.

Pharmacokinetics

Pharmacokinetics of metformin and chloroquine are monitored in order to evaluate a relationship between drug exposure, toxicity, and/or efficacy. Furthermore, the magnitude of the pharmacokinetic interactions between both compounds will be assessed. Blood samples will be taken at several time points during the study for the determination of the respective plasma levels.

The half-life of metformin is ±6.5 hours,[66] which means that with daily dosing the plasma level of metformin reaches a steady-state concentration within two days. The half-life of chloroquine is considerably longer (±2 weeks),[67] which means that with daily dosing the plasma level of chloroquine reaches a steady-state concentration within eight weeks in a flat-dosed scheme (which applies to patients who will have no tumour resection) and ±4-6 weeks under the proposed step-down dose scheme (see above).

Predose plasma samples (*i.e.* prior to study medication ingestion) will be taken on day 8 (week 2), day 29 (week 5) and every four weeks thereafter (see **Table 3**). Because chloroquine administration starts on day 8, the predose plasma sample on that day contains a metformin plasma concentration that reflects metformin monotherapy. The pharmacokinetic interaction between metformin and chloroquine is evaluated by comparing the metformin concentration on day 8 with the metformin concentration at subsequent time points. The relationship between exposure and toxicity is evaluated using all samples. The difference in the time after which steady-state serum levels of metformin and chloroquine are reached also help with distinguishing the source of any drug-related toxicity, because any toxicity in the first month is unlikely to be the result of chloroquine, but likely the result of metformin.

Detection of *D*-2HG levels in serum, urine, and/or bile

We will detect *D*-2HG levels in patient serum, urine, and/or bile using MS. Because our method distinguishes the *IDH1/2* mutation-specific *D*-2HG from the unspecific *L*-2HG, we expect a better signal-to-noise ratio and a higher sensitivity and specificity to detect *IDH1/2* mutations than in previous studies, where total 2HG levels were measured.[52 53 57] Bile samples will only be obtained from patients with intrahepatic cholangiocarcinoma with easy access to bile samples in the context of regular patient care, such as a percutaneous transhepatic biliary drain.

Detection of intratumoural 2HG levels

Intratumoural 2HG levels will be detected using long-echo MRS (PRESS) on a 3T MRI at the start and end of treatment of patients using protocols that were described before.[55] We will compare intratumoural 2HG levels before and after treatment to investigate whether MRS can be used to monitor therapy responses in *IDH1/2*-mutated solid tumours. We will also compare results from MRS with the results of DNA sequencing or immunohistochemistry to investigate whether MRS can be used to determine the mutational status of *IDH1/2* in patients with chondrosarcoma or intrahepatic cholangiocarcinoma.

Therapy response assessment

Response will be assessed by RECIST 1.1 guidelines[68] for chondrosarcoma and intrahepatic cholangiocarcinoma or RANO guidelines[69] for glioma on images obtained with CT and/or MRI scans. Scans will be performed at screening and every eight weeks from study inclusion. We will investigate whether NGS and MS analysis of ctDNA and plasma fractions, respectively, derived from blood samples that will be taken before, during and after the study treatment, can be used to monitor therapy responses. When there is pre-study and post-study primary tumour material available we will perform immunohistochemical staining with the appropriate IDH1/2 mutant-specific antibody to investigate the intratumoural mutational burden.

Toxicity monitoring

Patients will be interviewed for toxicity every 4 weeks and educated on frequently occurring side-effects of chloroquine and metformin (gastro-intestinal side-effects, signs of hypoglycaemia). Prolongation of QTc time is a rare adverse effect of chloroquine and patients will undergo an ECG every 24 weeks. Large cumulative doses (>460 gram) of chloroquine can induce retinopathy (Bull's Eye maculopathy).[70] Daily doses up to 250 mg per day for several years are considered to carry an acceptable risk for chloroquine-induced retinopathies.[71] In the proposed clinical trial, patients will be treated with 200 mg chloroquine per day (cumulative dose per year: 73 grams). Therefore, this clinical trial carries a very low risk to induce chloroquine-related retinopathies. Long-term use of chloroquine (>5 years or >300 grams cumulative dose) is an exclusion criterion for this trial to prevent chloroquine-related retinopathies. We will perform an ophthalmologic evaluation when the estimated lifetime chloroquine dose of a patient exceeds 300 grams during his/her trial participation.

Ethics and dissemination

This study is being conducted according to Good Clinical Practice guidelines as described in the International Conference on Harmonization Guideline E6 and in accordance with general ethical principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the medical-ethical review committee (METC) of the Academic Medical Centre, Amsterdam, the Netherlands and the Dutch national competent authority (CCMO) on 22 October 2015 under reference number NL53150.018.15.

A report describing the results of the study will be submitted to a peer-reviewed journal. Where permitted by patient data protection standards, data will be published and shared together with the publication of the study results.

An ethical limitation of the present clinical trial may be that the therapeutic index of metformin and chloroquine has been established in glioma and colorectal carcinoma cells,[16] but not in intrahepatic cholangiocarcinoma or chondrosarcoma models. However, this is primarily a dose-finding study. Follow-up phase II clinical trials will be rationally designed based on the pending evidence whether or not the efficacy of metformin and chloroquine treatment will be validated in model systems of other types of cancer by then.

LIST OF ABBREVIATIONS

αKG	alpha-ketoglutarate
AML	acute myeloid leukaemia
BBB	blood-brain barrier
b.i.d.	bis in die, two times a day
CTCAE	common terminology criteria for adverse events
ctDNA	circulating tumour DNA
D-2HG	*D*-2-hydroxyglutarate
DLT	dose-limiting toxicity
ETC	electron transport chain
IDH1/2	isocitrate dehydrogenase 1 or 2
$IDH1/2^{WT}$	IDH1/2 wild-type

IDH1/2^{MT}	IDH1/2 mutant
MAD	maximum administered dose
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
MTD	maximum tolerated dose
NGS	next-generation sequencing
q.d.	quaque die, one a day
RANO	response assessment in neuro-oncology
RD	recommended dose (for a phase II clinical trial)
RECIST	response evaluation criteria in solid tumours
TCA cycle	tricarboxylic acid cycle

DECLARATIONS

Consent for publication

Not applicable.

Availability of data and material

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests:

The authors declare no conflict of interest.

Funding:

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Authors' contributions

R.J.M. and J.W.W. conceived and designed the study. R.J.S., M.Kh., M.E.v.L., M.Ko., J.A.M.B., J.V.M.G.B., R.A.A., H.J.K, H.W.M.v.L., C.J.F.v.N., W.P.V., H.G. and T.M.v.G. guided the study design. R.J.M., M.Kh. and M.W.A.C. performed pilot study experiments. R.J.M. wrote the manuscript. All authors read and approved the final manuscript.

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Registration:

This article was registered at ClinicalTrials.gov under identifier NCT02496741.

Study dates:

Date of study registration: 30 June 2015

Date of ethical approval: 22 October 2015

Date of first enrollment: 17 November 2015

Patients included as of 25 October 2015: 3.

Expected date of enrollment completion: Q4 2017.

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FIGURE LEGENDS

Figure 1. Cellular carbohydrate and glutamine metabolism showing the pathways in which wild-type IDH1 and IDH2 are functional. Isocitrate dehydrogenase 1, 2, and 3 (IDH1/2/3) catalyse the conversion of isocitrate to α-ketoglutarate (αKG) in the cytoplasm and mitochondria, respectively, where IDH1 and IDH2 are NADPH-producing and IDH3 is NADH-producing. This reaction occurs in tricarboxylic acid (TCA) cycle(-like) metabolism and is fed by glucose influx and/or glutamine influx. The conversion of glutamine into αKG occurs in the glutaminolysis pathway and the last step is catalysed by glutamate dehydrogenase (GDH), which is inhibited by chloroquine and metformin. The TCA cycle produces NADH, which is used in the electron transport chain (ETC) and oxidative phosphorylation to generate ATP. Complex I of the ETC is inhibited by metformin. Wild-type IDH1/2 ($IDH1/2^{WT}$) differs from mutant IDH1/2 ($IDH1/2^{MUT}$) because the latter enzyme converts αKG into a novel oncometabolite, *D*-2-hydroxyglutarate (*D*-2HG).

Figure 2. Dosing schedule and study design for patients who will not undergo tumour resection. Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

Figure 3. Dosing schedule and study design for patients who have a tumour resection planned. Surgery will define the end of this time schedule (2 days before surgery). Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

TABLES AND LEGENDS

Table 1. Inclusion and exclusion criteria.

Key inclusion criteria
• Age ≥18 years. • Presence of a measurable intrahepatic cholangiocarcinoma or WHO grade II-III chondrosarcoma (RECIST 1.1 criteria [68]) or WHO grade II-IV glioma (RANO criteria [69]), both newly-diagnosed and refractory, relapsed, or recurrent tumours. • Tumour carries a *D*-2HG-generating mutation in *IDH1* or *IDH2* as determined by sequencing of primary tumour DNA, immunohistochemistry of primary tumour tissue with

an IDH1/2 mutant-specific antibody, or MRS imaging of the tumour (for glioma patients).

- ECOG/WHO performance status 0-2.
- Adequate renal function (creatinine <150 µmol/L or a creatinine clearance >60 ml/L).
- Adequate liver function (bilirubin <1.5 times the normal upper limit; ALAT and ASAT <2.5 the normal upper limit).
- Adequate bone marrow function (white blood cells >3.0 x 10^9/L, platelets >100 x 10^9/L).
- When patient is eligible for tumour resection, surgery is planned at least 4 weeks later than the start of study treatment.

Key exclusion criteria

- Concomitant other anti-cancer therapy (e.g. surgical resection, chemotherapy, targeted therapy, radiation therapy, surgery). Palliative therapy is permitted, such as:
  - palliative radiotherapy for symptomatic bone metastases,
  - dexamethasone for symptom relief in patients with glioma and cerebral oedema,
  - non-enzyme inducing anti-epileptic drugs (with the exception of topiramate) in patients with glioma and epileptic seizures.
- Severe and/or uncontrolled medical conditions at <6 months prior to randomization, such as:
  - unstable angina pectoris, symptomatic congestive heart failure, myocardial infarction or cardiac arrhythmias,
  - pulmonary insufficiency,
  - severe gastrointestinal, neurological (including epilepsy) or hematological diseases (interaction with chloroquine),
  - uncontrolled diabetes as defined by fasting serum glucose >12 mmol/l,
  - active or uncontrolled severe infection, including malaria,
  - cirrhosis, chronic active hepatitis or chronic persistent hepatitis.

- Serious concomitant systemic disorder that compromises the safety of the patient, at the discretion of the investigator.
- Patients who have a known history of alcohol abuse (interaction with metformin).
- Patients with known glucose-6-phosphate dehydrogenase deficiency, porphyria, myasthenia gravis or ocular/retinal aberrations (interactions with chloroquine).
- Patients who use digoxin, MAO inhibitors, fenylbutazone, oxygenbutazone, gold preparations or cimetidine (known pharmacokinetic interactions with chloroquine) or loop diuretics (known pharmacokinetic interaction with metformin) for which not a good alternative is available.
- Patients with a known hypersensitivity to metformin or chloroquine.
- Use of metformin or chloroquine in the previous 6 months or long-term use of chloroquine (>5 years or cumulative dose >300 grams) in the past.

Abbreviations: ctDNA, circulating tumour DNA; *D*-2HG, *D*-2-hydroxyglutarate; IDH1/2, isocitrate dehydrogenase 1 and 2; MRS, magnetic resonance spectroscopy; MS, mass spectrometry.

Table 2. Metformin dose escalation schedule.

Dose level	Dose of metformin given orally (total daily dose)	Minimum number of patients
-1	500 mg q.d. (500 mg total)	--
1 (starting)	500 mg b.i.d. (1000 mg total)	3
2	1000 mg b.i.d. (2000 mg total)	3
3	1500 mg b.i.d. (3000 mg total)	3

Abbreviations: b.i.d., two times a day; q.d., once a day.

Table 3. Timeline, study treatment, study visits and medical procedures.

Required investigations	Screening	Day 8 (week 2)	Day 29/week 5 and every 4 weeks thereafter	Day 57/week 9 and every 8 weeks thereafter	End of study
Visit number	1	2	3	4+	4+
Written informed consent	Prior to Screening				
Demographics (age, sex)	X				
Overall medical history	X				X
Physical examination, including weight and height	X		X	X	X
Vital signs (blood pressure, pulse)	X		X	X	X
ECOG/WHO performance status	X		X	X	X
CT or MRI scan of measurable lesion, ≤1 month prior to start treatment	X			X	X
Haematology	X		X	X	X

Serum chemistry:					
Hepatic function	X		X	X	X
Renal function	X		X	X	X
Glucose	X		X	X	X
HbA1c	X			X	X
Triglycerides	X			X	X
Cholesterol	X			X	X
Haemostatic parameters (aPPT and PT)	X				X
Insulin, IGF-1, IGF binding protein-3	X			X	X
Vitamin B12	X			X	X
Metformin concentration		X	X	X	X
Chloroquine concentration			X	X	X
MS of serum/urine/bile for *D*-2HG levels	X		X	X	X
MRS for intratumoural 2HG	X				X

levels					
Liquid biopsy	X		X	X	X
ECG	X				
Pregnancy test	X				
Optional: tumour biopsy	X			X	X

In addition to this scheme, an ECG will be performed every 24 weeks. Metformin and chloroquine concentrations will be taken at the end of study only when possible.

Abbreviations: (*D*-)2HG, (*D*-)2-hydroxyglutarate; IGF, insulin growth factor; MRS, magnetic resonance spectroscopy; MS, mass spectroscopy.

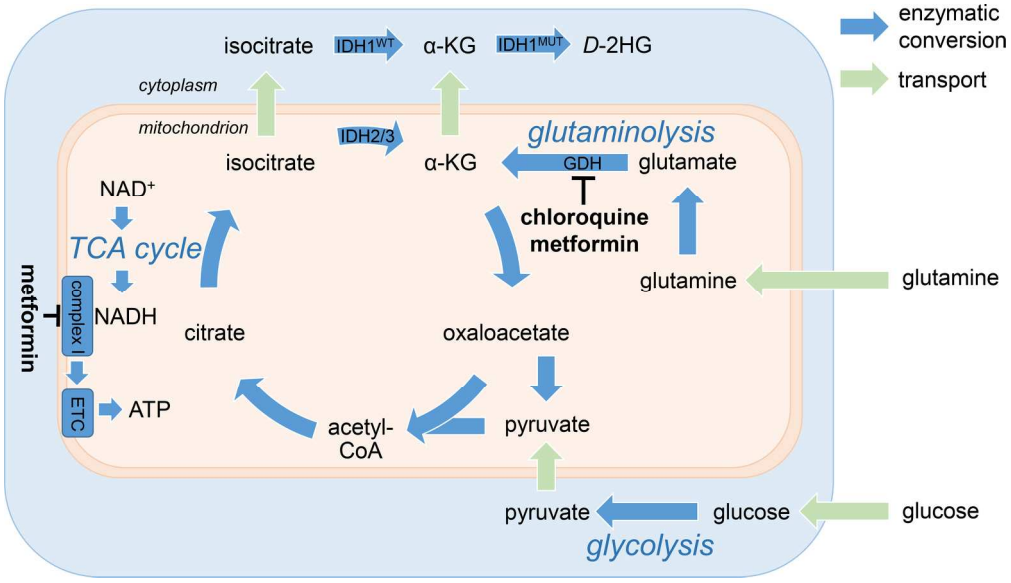

Figure 1. Cellular carbohydrate and glutamine metabolism showing the pathways in which wild-type IDH1 and IDH2 are functional. Isocitrate dehydrogenase 1, 2, and 3 (IDH1/2/3) catalyse the conversion of isocitrate to α-ketoglutarate (αKG) in the cytoplasm and mitochondria, respectively, where IDH1 and IDH2 are NADPH-producing and IDH3 is NADH-producing. This reaction occurs in tricarboxylic acid (TCA) cycle(-like) metabolism and is fed by glucose influx and/or glutamine influx. The conversion of glutamine into αKG occurs in the glutaminolysis pathway and the last step is catalysed by glutamate dehydrogenase (GDH), which is inhibited by chloroquine and metformin. The TCA cycle produces NADH, which is used in the electron transport chain (ETC) and oxidative phosphorylation to generate ATP. Complex I of the ETC is inhibited by metformin. Wild-type IDH1/2 (IDH1/2WT) differs from mutant IDH1/2 (IDH1/2MUT) because the latter enzyme converts αKG into a novel oncometabolite, D-2-hydroxyglutarate (D-2HG).

Figure 1
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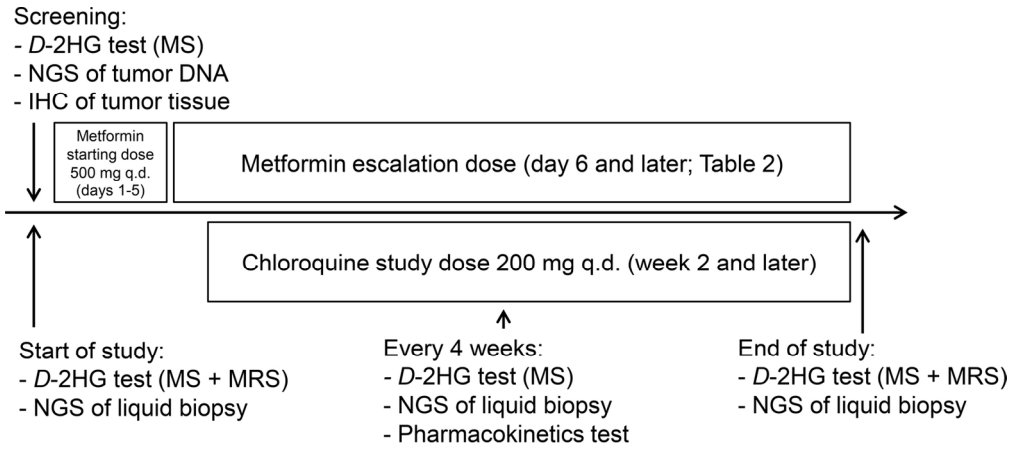

Figure 2. Dosing schedule and study design for patients who will not undergo tumour resection. Abbreviations: D-2HG, D-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.
Figure 2
75x32mm (600 x 600 DPI)

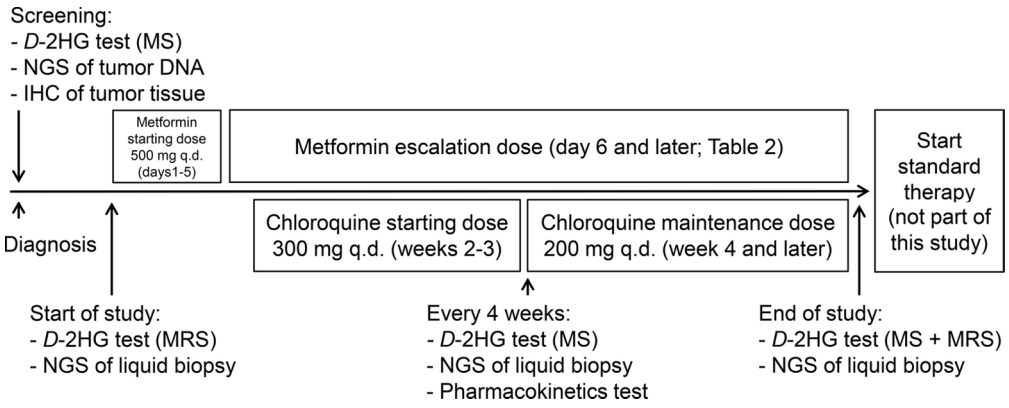

Figure 3. Dosing schedule and study design for patients who have a tumour resection planned. Surgery will define the end of this time schedule (2 days before surgery). Abbreviations: D-2HG, D-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

Figure 3
67x26mm (600 x 600 DPI)

Supplementary Table 1. Hematologic and non-hematologic dose-limiting toxicities.

Hematologic	Non-hematologic
• Absolute granulocyte count <0.5 x 10^9/l. • Febrile neutropenia (ANC <1.0 x 10^9/L, fever >38.5°C). • Platelets <25 x 10^9/l. • Bleeding due to thrombocytopenia, as determined by a physician.	• Diarrhoea > grade 3 despite optimal loperamide use. • Rash > grade 3 or grade 2 is medically concerning or unacceptable to the patient. • Other grade 3 effects considered to be treatment related. • Missing >7 days of treatment for toxicity reasons.

Grading of side effects is performed using CTCAE. Abbreviations: ANC, absolute neutrophil count.

SUPPLEMENTARY FILE 1

3+3 dose-escalation schedule and intrapatient dose-escalation

Maximum administered dose

If 0/3 patients exhibit dose-limiting toxicity at this dose level:

- Dose escalation to the next dose level may begin in a new cohort of patients
- Patients enrolled on the previous dose level who are still receiving therapy may now undergo intrapatient dose escalation to this new dose level provided that they have experienced no drug related toxicity of grade 2 or higher at the previous dose level.

If 1/3 patients exhibit dose-limiting toxicity at this dose level:

- Expand dose level to a total of six patients. Toxicity information from patients who underwent intrapatient dose escalation can be used for expansion cohorts, but only when they have completed at least 8 weeks of treatment at the new dose level.
- If no further DLT events are observed, dose escalation to the next dose level may begin in a new cohort of patients and patients enrolled on the previous dose level who are still receiving therapy may now undergo intrapatient dose escalation to this dose level provided that they have experienced no drug related toxicity of grade 2 or higher.
- If further DLTs are observed (i.e. in ≥2/6 patients), this dose level will be considered the maximum administered dose (MAD).

If ≥2/3 patients exhibit dose-limiting toxicity

- This dose level will be considered the MAD.
- If this toxicity occurs at level 1 (starting level), dose de-escalation to level -1 will be applied.

Recommended phase II dose

As described in the full text manuscript, the MAD is the dose in which ≥2/3 or ≥2/6 patients experience a DLT, or the final dose from the dose escalation schedule (1500 mg metformin b.i.d. and 200 mg chloroquine q.d.). One dose level below the MAD will be considered the RD for follow-up phase II clinical trials. When the starting dose level (“1”) is the MAD, we will de-escalate the dose level to dose level “-1”. When we do not observe DLTs in three patients or one DLT in six patients at this dose level, then dose level “-1” will be the RD. When we observe more than one DLT, the combination of metformin and chloroquine will be considered too toxic to be useful in cancer patients. In contrast to this situation where we have to accept the lowest dose-escalation level as the RD, when 0/6 patients experience DLTs at the final dose level of the dose escalation schedule (i.e. dose level “3”), this can be considered the RD for follow-up phase II clinical trials, instead of dose level “2”.

Up to a total of six patients may be treated at the RD level to assure information on the safety profile when that dose is complete. When clinically appropriate, intermediate dose levels may be studied to assure that the RD is the highest tolerable. Furthermore, when pharmacokinetic data suggests that saturating absorption of drug is occurring on a b.i.d. oral administration

level, further dose splitting to three times a day or four times a day schedules may be considered.

Patient replacement

Three patients within a dose level must be observed for eight weeks before accrual to the next dose level may begin. If a patient is withdrawn from the study prior to completing 22 days of therapy without experiencing a DLT prior to withdrawal, an additional patient may be added to that dose level. Patients missing seven or more doses (one week) due to toxicity will not be replaced since these patients will be considered to have experienced a dose-limiting toxicity.

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	24
	2b	All items from the World Health Organization Trial Registration Data Set	1,12-14,20,21,23,24,
Protocol version	3	Date and version identifier	All pages of protocol
Funding	4	Sources and types of financial, material, and other support	23
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1,23,24
	5b	Name and contact information for the trial sponsor	1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	23,24

	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N/A
Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-11
	6b	Explanation for choice of comparators	4-11
Objectives	7	Specific objectives or hypotheses	10-13
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	14-16
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	22 of protocol
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	30-31
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	32-34
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	15
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	N/A
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	30

Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	13-14
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	32-34, fig 2-3
Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	15
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	N/A
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	N/A
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	N/A
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	N/A
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A
Methods: Data collection, management, and analysis			

Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	32-34
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	32-34
Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	44 of protocol
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	40 of protocol
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	N/A
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	N/A
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	45-46 of protocol
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	47-48 of protocol
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	45-46 of protocol
Ethics and dissemination			

Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	3
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	N/A
Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	49 of protocol
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	49 of protocol
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	45 of protocol
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	23
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	45-46 of protocol
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	60 of protocol
	31b	Authorship eligibility guidelines and any intended use of professional writers	60 of protocol
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	60 of protocol
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	N/A

Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	42-43 of protocol

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated.

BMJ Open

Study protocol of a phase Ib/II clinical trial of metformin and chloroquine in patients with *IDH1*-mutated or *IDH2*-mutated solid tumors

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STUDY PROTOCOL OF A PHASE IB/II CLINICAL TRIAL OF METFORMIN AND CHLOROQUINE IN PATIENTS WITH *IDH1*-MUTATED OR *IDH2*-MUTATED SOLID TUMOURS

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ABSTRACT

Introduction: High-grade chondrosarcoma, high-grade glioma, and intrahepatic cholangiocarcinoma are aggressive types of cancer with a dismal outcome. This is due to the lack of effective treatment options, emphasizing the need for novel therapies. Mutations in the genes *IDH1* and *IDH2* occur in 60% of chondrosarcoma, 80% of WHO grade II-IV glioma and 20% of intrahepatic cholangiocarcinoma. *IDH1/2*-mutated cancer cells produce the oncometabolite *D*-2-hydroxyglutarate (*D*-2HG) and are metabolically vulnerable to treatment with the oral antidiabetic metformin and the oral antimalarial drug chloroquine.

Methods and analysis: We describe a dose-finding phase Ib/II clinical trial, in which patients with *IDH1/2*-mutated chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma are treated with a combination of metformin and chloroquine. Dose escalation is performed according to a 3+3 dose-escalation scheme. The primary objective is to determine the maximum tolerated dose to establish the recommended dose for a phase II clinical trial. Secondary objectives of the study include (1) determination of pharmacokinetics and toxic effects of the study therapy, for which metformin and chloroquine serum levels will be determined over time; (2) investigation of tumour responses to metformin plus chloroquine in *IDH1/2*-mutated cancers using CT/MRI scans; and (3) whether or not tumour responses can be measured by non-invasive *D*-2HG measurements (mass spectrometry (MS) and magnetic resonance spectroscopy (MRS)) of tumour tissue, serum, urine, and/or bile or next-generation sequencing of circulating tumour DNA (liquid biopsies). This study may open a novel treatment avenue for *IDH1/2*-mutated high-grade chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma by repurposing the combination of two inexpensive drugs that are already approved for other indications.

Ethics and dissemination: This study has been approved by the medical-ethical review committee of the Academic Medical Center, Amsterdam, The Netherlands. The report will be submitted to a peer-reviewed journal.

STRENGTHS AND LIMITATIONS OF THIS STUDY

Strengths:

- To the best of our knowledge, this is the first clinical trial in that investigates the combination of metformin and chloroquine in cancer patients.
- Tumor responses to the study therapy will be monitored using conventional CT/MRI scans and using magnetic resonance spectroscopy or serum mass spectrometry for *D*-2HG levels.

Limitations:

- Because this is primarily a dose-finding study, we may not be able to study the efficacy of metformin and chloroquine.
- When patients do not consent to tumour biopsies/re-resections, this diminishes the possibility for translational analyses.

ARTICLE MANUSCRIPT

Introduction

IDH1 and IDH2 are homodimeric enzymes that reversibly convert isocitrate to α-ketoglutarate (αKG) in cytoplasm and mitochondria, respectively. Somatic heterozygous mutations in *IDH1/2* that produce the oncometabolite *D*-2-hydroxyglutarate (*D*-2HG) are observed in substantial percentages of various tumour types such as chondrosarcoma (60%), WHO grade II-III glioma (80%), secondary WHO grade IV glioblastoma (80%), and intrahepatic cholangiocarcinoma (20%).[1] In addition, *IDH1/2* mutations occur in varying percentages of acute lymphocytic leukaemia (10%), acute myeloid leukaemia (AML; 20%), angioimmunoblastic T-cell lymphoma (40%), colorectal cancer (5%), and melanoma (12%).[1] In chondrosarcoma and glioma, *IDH1/2* mutations are considered very early or even inaugural genetic defects, and are thus present in a large fraction of, or even all, cancer cells.[2 3] This renders *IDH1/2* mutations an interesting target for anti-cancer treatment because such tumour homogeneity decreases the risk of therapy resistance.[4] Recently, inhibitors of mutant IDH1 and IDH2 were developed that may be effective in stalling malignant progression of early-stage *IDH1/2*-mutated cancers.[5 6]

Prognosis and therapeutic options of cancers in which *IDH1/2* mutations occur

The prognosis of solid tumours with frequent occurrence of *IDH1/2* mutations remains poor. The current standard therapy for chrondrosarcoma is surgery. There is no evidence for a benefit of (adjuvant) radiotherapy or chemotherapy, as chondrosarcoma are considered to be highly therapy resistant.[7] Consequently, the 1-year survival rate of metastasized high-grade chondrosarcoma is <10%.[8]Gliomas vary from WHO grade II diffuse astrocytoma and diffuse oligodendroglioma, with median survivals of more than five years,[9] to WHO grade IV glioblastoma, with a median survival of only 15 months despite aggressive treatment using

radiotherapy and temozolomide.[10] Gliomas are diffusely growing tumours, which renders surgery ineffective, emphasizing the dire need for novel therapies. Furthermore, the blood-brain barrier (BBB) prohibits the use of most chemotherapeutics and the surrounding normal brain hampers aggressive radiotherapy regimens due to limitations that are raised by healthy brain tissue.[11] Intrahepatic cholangiocarcinoma is resectable in only 40% of patients.[12] In unresectable cases, intrahepatic cholangiocarcinoma patients are offered palliative treatment as standard of care with the chemotherapy combination of cisplatin and gemcitabine, with a median overall survival of 11.7 months.[13]

Metabolic effects of *IDH1/2* mutations

Heterozygous hotspot *IDH1/2* mutations disable IDH1/2 wild-type enzyme activity[14-16] and induce a neo-enzymatic activity that leads to the production and subsequent accumulation of *D*-2HG.[17-19] *D*-2HG is normally present only in trace amounts in normal tissues and cells but accumulates up to 50 mM in *IDH1/2*-mutated glioma.[17] *D*-2HG is chemically very similar to α-ketoglutarate (αKG) and inhibits over 60 αKG-dependent enzymes, resulting in global DNA/histone hypermethylation, decreased hypoxia-inducible factor 1a (HIF1α) expression, and perturbed collagen maturation.[1] Depending on the cellular context, these effects are the basis of oncogenesis and imply a dependence on *D*-2HG of early-stage *IDH1/2*-mutated tumours.[1]

IDH1/2-mutated cancer cells need αKG to synthesize *D*-2HG and fuel the tricarboxylic acid (TCA) cycle to support their metabolism. αKG is generated by glycolysis (glucose breakdown) or glutaminolysis (glutamine/glutamate breakdown).[20] *IDH1/2* mutations

downregulate αKG levels by consuming αKG and by inhibition of αKG production via direct effects, *i.e.* by disabling *IDH1/2* wild-type kinetics, and indirect effects, *e.g.* by decreasing TCA cycle activity.[1] Therefore, *IDH1/2*-mutated cancer cells rely on glutaminolysis for sufficient αKG supply to generate the oncometabolite *D*-2HG (**Figure 1**).[21] The conversion of glutamate to αKG is catalysed by glutamate dehydrogenase (GDH), which is the final step of glutaminolysis and can be inhibited by the anti-malaria drug chloroquine and the antidiabetic drug metformin.[20 22-24] In addition, *IDH1*-mutated glioma cells show increased levels of autophagy, likely as a survival mechanism of cells to metabolic stress by catabolizing proteins in order to provide substrates for energy production in stress/starvation contexts.[25] Autophagy is inhibited by chloroquine[26] and the anti-cancer properties of chloroquine may thus be selective for *IDH1/2*-mutated cells because it inhibits glutaminolysis and autophagy on which the cells are dependent.

IDH1/2 mutations induce further metabolic stress in *IDH1/2*-mutated cancer cells via inhibition of the TCA cycle and electron transport chain (ETC) by *D*-2HG. More specifically, *D*-2HG inhibits enzymatic activity of complex IV (cytochrome C oxidase) of the ETC[27] and the TCA(-like) enzymes IDH1/2 and αKG dehydrogenase.[16] This reduces oxidative phosphorylation, the primary source of ATP in cancer cells.[27 28] This metabolic stress is amplified *in vitro* in *IDH1*-mutated glioma and colorectal carcinoma cells using compounds that inhibit ETC complex I, such as the oral antidiabetic biguanide metformin and phenformin, which selectively restrict the proliferation of these cells.[16 28]

Another metabolic vulnerability of *IDH1/2*-mutated glioma may be their excess deposition of the acidic *D*-2HG in their microenvironment, which is hypothesized to contribute to their diffuse growth.[29 30] Chloroquine buffers the tumor[31] and may decrease this acidification, reduce the diffuse growth and ultimately increase the treatability of *IDH1/2*-mutated glioma.

Metabolism of *IDH1/2*-mutated tumours as therapeutic target

Metformin and chloroquine increase metabolic stress in *IDH1/2*-mutated cells, as is described above. Patients with *IDH1/2*-mutated glioblastoma have a prolonged survival and better radiotherapy/chemotherapy response when compared with *IDH1/2* wild-type counterparts,[14 32 33] while in chondrosarcoma a correlation between mutation and survival was absent.[2] We and others have shown that *IDH1/2* mutations sensitize glioma and colorectal carcinoma cells to therapies that involve oxidative stress, such as radiotherapy, cisplatin, and carmustine.[16 34 35] Combined, these data suggest that at least some types of cancer with *IDH1/2* mutations should be targeted by compounds that exploit this presumed metabolic vulnerability rather than compounds that decrease metabolic stress (*i.e.* IDH1/2-mutant inhibitors). Accordingly, we hypothesized that the difference in survival of patients with *IDH1/2*-mutated glioma or intrahepatic cholangiocarcinoma versus *IDH1/2* wild-type counterparts is caused by dysregulation of cellular defence mechanisms by *IDH1/2* mutations against anti-cancer therapy.[1 16 36] Little is known about the role of *IDH1/2* mutations in late-stage cancer. It is plausible that with increasing mutational burden, the dependence of late-stage malignant tumours on *IDH1/2* mutations decreases, diminishing the therapeutic index of IDH1/2-mutant inhibitors.[37 38] On the other hand, metabolic stress that results from *IDH1/2* mutations persists, and this metabolic vulnerability provides an excellent target for therapy irrespective of the tumour stage.

Discussion regarding the use of metformin and chloroquine

Metformin and chloroquine are readily-available, inexpensive, and safe drugs that are already FDA/EMA-approved for other indications. The safety profiles of metformin and

chloroquine are favourable over other anti-cancer modalities, which may aid rapid implementation of these drugs into therapies for patients with *IDH1/2*-mutated cancers. A caveat is that the combined safety of metformin and chloroquine is to be proven by our study, although there are no reports of toxic side-effects of this combination in the literature whereas the prevalence of both diabetes and malaria is high. Since both drugs are off patent, combination treatment with metformin and chloroquine can become a therapeutic advance for patients with *IDH1/2*-mutated solid tumours that is considerably less expensive than products of other anti-cancer research efforts. The potential of metformin and chloroquine as adjuvant drugs was recently demonstrated *in vivo,* where metformin or chloroquine had a sensitizing and/or synergistic anti-tumour effect in combination with temozolomide,[39 40] cisplatin,[41 42] and gemcitabine[43 44] in xenograft models or proof-of-concept clinical trials of various types of human cancer, including glioma. Metformin, but not chloroquine, sensitized xenograft models of various types human cancer to ionizing radiation.[45 46]

Possible concerns may be related to the bioavailability of metformin. We have observed high expression of metformin transporters in chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma cell lines and primary tissue (OCT1-3; The Cancer Cell Line Encylopedia[47] and our own unpublished data). Therefore, we expect to achieve sufficient intratumoural metformin concentrations with dose levels 2 and 3 of our dose-escalation protocol. Whereas millimolar metformin concentrations are necessary to activate the necessary antineoplastic cellular targets *in vitro,* these targets were already activated at ±300-fold lower metformin concentrations *in vivo*. When metformin fails to show any metabolic or anti-tumour effect, we may investigate the feasibility of phenformin treatment in future studies. Phenformin is the lipophilic analogue of metformin which does not depend on transporters to enter cells. However, phenformin has a less favourable safety compared with metformin because it carries an increased risk of inducing lactic acidosis. As a consequence,

phenformin approval for the treatment of diabetes mellitus type 2 was withdrawn by the FDA and EMA in the 1970s[48] and in contrast to metformin, phenformin is not readily available.

With respect to chloroquine, possible concerns may be related to the plethora of cellular targets of chloroquine. Inhibition of autophagy and glutaminolysis and buffering of the tumour milieu are the potential therapeutic targets of chloroquine in *IDH1/2*-mutated cancers. Besides these, chloroquine also induces apoptosis and affects the body's immune response to the tumour *in vitro* and/or *in vivo* at concentrations that may be achieved using the dose that we use in the present clinical trial.[31] These properties of chloroquine as a "dirty drug" may lead to toxicity problems.

For the treatment of glioma, adequate drug penetration of the BBB is necessary for relevant tumour responses. Notwithstanding that high-grade glioma often destruct the BBB, *in vivo* experiments in mice have shown that metformin and chloroquine adequately pass the blood-brain barrier.[49 50]

Non-invasive detection of *IDH1/2* mutations

The gold standard of *IDH1/2* mutation detection is genetic analysis of tumour DNA. In glioma, 90% of all *IDH1/2* mutations are $IDH1^{R132H}$ and its presence can be reliably detected using a immunohistochemistry of glioma tissue with an $IDH1^{R132H}$-specific antibody.[51] The presence of *IDH1/2* mutations in AML[52] and intrahepatic cholangiocarcinoma[53] can be easily, reliably, and non-invasively detected via determination of 2HG levels or *D*-2HG levels in serum or urine by mass spectrometry (MS). Furthermore, MS-determined 2HG serum levels correlate with therapy response in these cancers.[52 53] In a previous study investigating intrahepatic

cholangiocarcinoma, total 2HG levels in serum predicted the presence of an *IDH1/2* mutation (as determined using targeted DNA sequencing) with a sensitivity of 83% and a specificity of 90%.[53]

Whereas no non-invasive detection methods of *IDH1/2* mutations have been described to be effective in chondrosarcoma yet, the presence of *IDH1/2* mutations in glioma can be determined using magnetic resonance spectroscopy (MRS) of the brain, which detects intratumoural 2HG levels.[54 55] Conversely, serum 2HG levels correlate poorly with the *IDH1/2* mutational status in glioma due to a limited blood-brain barrier passage of *D*-2HG.[56] Urine 2HG levels are higher in patients with *IDH1*-mutated glioma than in patients with *IDH1* wild-type glioma,[57] although another study reported decreased 2HG levels in the urine of patients with *IDH1*-mutated glioma and showed that the ratio of serum 2HG levels to urine 2HG levels is most predictive for the *IDH1* mutational status in glioma.[58] Most aforementioned measurements determined total 2HG levels and thus did not discriminate between the *D*-enantiomer of 2HG (which is specific for *IDH1/2* mutations) and the *L*-enantiomer of 2HG (which is unspecific and is generated during hypoxia).[59 60] Better separation of *D*-2HG and *L*-2HG may allow for *IDH1/2* mutational status predictions with higher sensitivity and specificity.

Besides methods that detect *D*-2HG accumulation, *IDH1/2* mutations may also be detected via next-generation sequencing (NGS) of circulating tumour DNA (ctDNA) that is isolated from serum as liquid biopsies. Liquid biopsies contain a collection of ctDNA sequences which is representative for the heterogeneity of the tumour. Therefore, liquid biopsies are more informative than tissue biopsies, which are subject to selection bias as a result of the tumour heterogeneity. In liquid biopsies, variant allelic frequencies can be used as biomarkers for tumour load and dynamic clonal hierarchies within the tumour.[61]

Hypothesis and outlook

To summarize, fundamental and translational research by us and others revealed that *IDH1/2* mutations impart therapeutically targetable metabolic vulnerabilities to cells from several types of cancer.[16 20 21 27 28] We aim to use these metabolic alterations in *IDH1/2*-mutated tumours for screening purposes and tumour response monitoring purposes using non-invasive modalities. Furthermore, we aim to specifically inhibit the metabolic processes that are essential to *IDH1/2*-mutated tumours using metformin and chloroquine, which specifically target the metabolic vulnerabilities that are caused by *IDH1/2* mutations.

We hypothesize that metformin and chloroquine can be safely used as anti-cancer drugs for patients with *IDH1/2*-mutated chondrosarcoma, glioma, and intrahepatic cholangiocarcinoma and that tumour response to treatment can be monitored by measuring tumour size and/or levels of *D*-2HG in serum, urine, bile, and/or the tumoural mass. This hypothesis will be tested in a phase Ib/II clinical trial. There are no reports of clinical trials of combined treatment with metformin and chloroquine yet. In the future, metformin and chloroquine may be used as stand-alone therapy for patients with *IDH1/2*-mutated cancers, especially in chondrosarcoma for which no effective therapies beside surgery exists, or besides conventional anti-cancer treatments such as radiation and temozolomide in glioma and cisplatin and gemcitabine in intrahepatic cholangiocarcinoma.

Methods and analysis

Overall study design

MACIST is a nonrandomized, open-label, dose-finding, multi-centre phase Ib/II clinical trial with a combined regimen of metformin and chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma. Drug dosing will follow a 3+3 dose escalation scheme. Patients will be enrolled at three academic hospitals in The Netherlands (Academic Medical Centre and VU University Medical Centre, both in Amsterdam and the Leiden University Medical Centre in Leiden).[62]

Objectives

Primary objective

To determine the maximum tolerated dose (MTD) and recommended dose (RD) of metformin plus chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma.

Secondary objectives

- To describe the toxic effects and pharmacokinetics of metformin plus chloroquine in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma;
- To provide evidence of complete or partial tumour regression in patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma after treatment with metformin plus chloroquine;
- To provide evidence that the *IDH1/2* mutational status of chondrosarcoma, glioma and intrahepatic cholangiocarcinoma can be assessed using enantiomer-specific measurements that determine the separate *D*-2HG and *L*-2HG levels in serum, urine,

or bile (with better sensitivity and specificity than with measurements that determine total 2HG concentrations);

- To provide evidence that the *IDH1/2* mutational status of chondrosarcoma and intrahepatic cholangiocarcinoma patients can be determined by MRS-facilitated detection of intratumoural 2HG levels or liquid biopsies;
- To provide evidence of activity of metformin plus chloroquine related to *D*-2HG levels in the serum, urine, bile, and/or tumoural mass of patients with *IDH1/2*-mutated chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma.

Trial end points

Primary end points (outcomes)

- We will determine the MTD, which is the chloroquine plus metformin dose in which ≤1 in three patients (of a 3+3 dose-escalation schedule) show serious adverse effects.
- We will determine the RD of chloroquine plus metformin, which is the dose level one step below the MTD.

Secondary end points (outcomes)

- Serum metformin and chloroquine concentrations will be measured to investigate the pharmacokinetics of this combination and establish a relationship or not between drug exposure and toxicity and/or efficacy.

- Tumour size will be measured using a MRI and/or CT scan before and after treatment with metformin plus chloroquine to monitor tumour response, using response evaluation criteria in solid tumours (RECIST) 1.1 in chondrosarcoma and intrahepatic cholangiocarcinoma patients and response assessment in neuro-oncology (RANO) in glioma patients.
- *D*-2HG concentrations in serum, urine, bile, and/or the tumoural mass will be measured by MS every four weeks during treatment and by MRS at the start and end of the treatment to investigate the effects of metformin plus chloroquine on *D*-2HG levels. Furthermore, these *D*-2HG measurements will be compared with results obtained from CT and/or MRI scans to investigate whether determinations of *D*-2HG concentrations in serum, urine, bile, and/or the tumoural mass correlate with radiologically observed tumour responses to therapy.
- The variant allelic frequency of *IDH1* mutations or *IDH2* mutations will be measured using NGS on liquid biopsies at the start and end of the treatment and every four weeks during treatment to determine the effects of metformin plus chloroquine on the variant allelic frequency and mutational load of these mutations.

Participants

In brief, this trial will enrol eligible patients with *IDH1/2*-mutated and newly-diagnosed, recurrent, relapsed or refractory and/or metastasized WHO grade II-III chondrosarcoma,[63] WHO grade II-IV glioma,[64] or intrahepatic cholangiocarcinoma. All inclusion and exclusion criteria are listed in **Table 1**. The trial will enrol patients who have no tumour resection planned (**Figure 2**) and those who have a tumour (re-)resection planned (**Figure 3**). These patients will be studied in their waiting period until resection (approximately 6-8 weeks). We are especially interested in patients that had a tumour resection in the past of which tumour

material is available, who had a recurrence of their tumour and who will have a re-resection of this recurrent tumour, because we will then be able to collect pre- and post-treatment samples of these patients. This may also be achieved using sequential tumour biopsies. For patients that have no tumour resection planned, the end of the study is defined as when a patient chooses to withdraw from the study, when a patient experiences a DLT, or when tumour progression occurs. For patients who will have a tumour resection, the study will be conducted during the waiting period until surgery. The end of study is defined similarly as for patients that have no tumour resection planned or two days before surgery.

This phase Ib/II dose-finding study has three dose-escalation levels. According to a 3+3 dose-escalation scheme, we need a maximum of 18 patients (a maximum of 6 patients in 3 dose escalation levels). A maximum of 10 patients can be enrolled of each tumour type (chondrosarcoma, glioma, or intrahepatic cholangiocarcinoma).

Dose of study drugs and dose escalation schedule

Metformin

The starting dose of metformin will be 500 mg per os q.d. during the first five days. Subsequently, the metformin dose will be escalated as outlined in **Table 2**. This escalation schedule is based on an earlier phase II clinical trial in pancreatic adenocarcinoma.[65] The purpose of the lower metformin starting dose is to reduce side effects of metformin, especially gastro-intestinal side effects. This starting dose mimics dosage schedules of metformin treatment in patients with type 2 diabetes mellitus.

Chloroquine

Chloroquine will be added to metformin in week 2 of the study and chloroquine doses will not be escalated. Patients who have no tumour resection planned will be treated with 200 mg chloroquine q.d. For patients who have a tumour resection planned, chloroquine will be given in a step-down dosing schedule. The starting dose (first two weeks of chloroquine administration; week 2 and 3 of study) is 300 mg q.d. In subsequent weeks (week 4 of the study and later), the chloroquine maintenance dose will be 200 mg q.d. Because we expect the study duration to be a few weeks in patients with resectable tumours (there usually is a waiting time of 6-8 weeks from diagnosis until surgery), the higher starting dose in patients with resectable tumours allows build-up of functional chloroquine serum concentrations in a shorter time, thereby increasing the chance of a measurable effect within the period of time in which the study will be conducted. This dosing schedule is necessary because of the long half-life of chloroquine. Step-down dosing schedules of chloroquine are also used in systematic lupus erythematodes.[66]

Dose finding

The rate of subject entry and escalation to the next dose level will depend upon assessment of the safety profile of patients entered at the previous dose level. Toxicity will be evaluated according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.03. A minimum of 3 patients will be entered on each dose level. **Supplementary File 1** describes the standard 3+3 dose-escalation schedule of our study, the procedures for intrapatient dose-escalation and patient replacement and the finding of the recommended phase II dose. Dose (de)escalation will be based on the toxicity assessment in the first eight weeks of therapy and the documentation of any dose-limiting toxicities (DLTs). To be considered as a DLT, the toxicity must be considered to be related to the study drug. DLTs are defined in **Supplementary Table 1.** When a patient experiences a DLT, he/she can

decide to withdraw from the study or go into intrapatient de-escalation by receiving metformin at one dose level lower than the dose level that provoked the DLT.

Screening for *IDH1/2* mutations

The *IDH1/2* mutational status of patients will be assessed using DNA sequencing or immunohistochemistry. In a patient with glioma, the presence of an *IDH1/2* mutation can also be established using MRS to detect intratumoural 2HG levels.[54 55]

Study visits

Patients with *IDH1/2*-mutated tumours will visit their hospital of inclusion once for additional eligibility screening (see in- and exclusion criteria). Once enrolled in the study, patients will undergo a study visit after one week, in which blood will be drawn for pharmacokinetic analysis (see below) and after four weeks, in which blood will be drawn for serum *D*-2HG MS analysis, for analysis of hematologic, hepatic, renal, and chemistry parameters and for further pharmacokinetic analyses. Every eight weeks, these patients will have a more elaborate study visit in which they will undergo a CT/MRI scan in addition to the procedures that will also occur at study visits every four weeks. Specifics for each study visit are shown in **Table 3**.

Pharmacokinetics

Pharmacokinetics of metformin and chloroquine are monitored in order to evaluate a relationship between drug exposure, toxicity, and/or efficacy. Furthermore, the magnitude of the pharmacokinetic interactions between both compounds will be assessed. Blood samples will be taken at several time points during the study for the determination of the respective plasma levels.

The half-life of metformin is ±6.5 hours,[67] which means that with daily dosing the plasma level of metformin reaches a steady-state concentration within two days. The half-life of chloroquine is considerably longer (±2 weeks),[68] which means that with daily dosing the plasma level of chloroquine reaches a steady-state concentration within eight weeks in a flat-dosed scheme (which applies to patients who will have no tumour resection) and ±4-6 weeks under the proposed step-down dose scheme (see above).

Predose plasma samples (*i.e.* prior to study medication ingestion) will be taken on day 8 (week 2), day 29 (week 5) and every four weeks thereafter (see **Table 3**). Because chloroquine administration starts on day 8, the predose plasma sample on that day contains a metformin plasma concentration that reflects metformin monotherapy. The pharmacokinetic interaction between metformin and chloroquine is evaluated by comparing the metformin concentration on day 8 with the metformin concentration at subsequent time points. The relationship between exposure and toxicity is evaluated using all samples. The difference in the time after which steady-state serum levels of metformin and chloroquine are reached also help with distinguishing the source of any drug-related toxicity, because any toxicity in the first month is unlikely to be the result of chloroquine, but likely the result of metformin.

Detection of *D*-2HG levels in serum, urine, and/or bile

We will detect *D*-2HG levels in patient serum, urine, and/or bile using MS. Because our method distinguishes the *IDH1/2* mutation-specific *D*-2HG from the unspecific *L*-2HG, we

expect a better signal-to-noise ratio and a higher sensitivity and specificity to detect *IDH1/2* mutations than in previous studies, where total 2HG levels were measured.[52 53 57] Bile samples will only be obtained from patients with intrahepatic cholangiocarcinoma with easy access to bile samples in the context of regular patient care, such as a percutaneous transhepatic biliary drain.

Detection of intratumoural 2HG levels

Intratumoural 2HG levels will be detected using long-echo MRS (PRESS) on a 3T MRI at the start and end of treatment of patients using protocols that were described before.[55] We will compare intratumoural 2HG levels before and after treatment to investigate whether MRS can be used to monitor therapy responses in *IDH1/2*-mutated solid tumours. We will also compare results from MRS with the results of DNA sequencing or immunohistochemistry to investigate whether MRS can be used to determine the mutational status of *IDH1/2* in patients with chondrosarcoma or intrahepatic cholangiocarcinoma.

Therapy response assessment

Response will be assessed by RECIST 1.1 guidelines[69] for chondrosarcoma and intrahepatic cholangiocarcinoma or RANO guidelines[70] for glioma on images obtained with CT and/or MRI scans. Scans will be performed at screening and every eight weeks from study inclusion. We will investigate whether NGS and MS analysis of ctDNA and plasma fractions, respectively, derived from blood samples that will be taken before, during and after the study treatment, can be used to monitor therapy responses. When there is pre-study and post-

study primary tumour material available we will perform immunohistochemical staining with the appropriate IDH1/2 mutant-specific antibody to investigate the intratumoural mutational burden.

Toxicity monitoring

Patients will be interviewed for toxicity every 4 weeks and educated on frequently occurring side-effects of chloroquine and metformin (gastro-intestinal side-effects, signs of hypoglycaemia). Prolongation of QTc time is a rare adverse effect of chloroquine and patients will undergo an ECG every 24 weeks. Large cumulative doses (>460 gram) of chloroquine can induce retinopathy (Bull's Eye maculopathy).[71] Daily doses up to 250 mg per day for several years are considered to carry an acceptable risk for chloroquine-induced retinopathies.[72] In the proposed clinical trial, patients will be treated with 200 mg chloroquine per day (cumulative dose per year: 73 grams). Therefore, this clinical trial carries a very low risk to induce chloroquine-related retinopathies. Long-term use of chloroquine (>5 years or >300 grams cumulative dose) is an exclusion criterion for this trial to prevent chloroquine-related retinopathies. We will perform an ophthalmologic evaluation when the estimated lifetime chloroquine dose of a patient exceeds 300 grams during his/her trial participation.

Statistical methods

The patient sample size in the clinical trial (n = 20) is based on the 3+3 dose-escalation schedule and the three proposed dose-escalation steps. With 20 patients, we are able to determine whether dose level 3 is the MTD, even when we need a 3+3 expansion cohort at step 2 and step 3 and when 25% of patients are not evaluable because the patients discontinued their study participation before completing 4 weeks of study treatment. Tumor

volumes (from CT/MRI scans), serum metformin, chloroquine and *D*-2HG concentrations (from MRS/MS measurements) and *IDH1/2* mutational loads (from NGS) from before, during and after treatment time points will be compared using the paired samples *t*-test.

Data management, auditing and access

Source data from the trial will be locally stored and entered in electronic case report forms. Based on the guidelines by the NFU (Dutch Federation of University Medical Centers) the risk of this study was qualified as 'moderate'. According to this, a 'minimal intensive auditing' is advised, which will be performed by an independent clinical research associate (for details, see **Supplementary File 1**). Besides this clinical research associate, only the investigators are allowed access to the source data. As part of informed consent, patients will be informed as to the strict confidentiality of their patient data, but that their medical records may be reviewed for trial purposes by clinical research associates. Being primarily a phase Ib dose-finding study, this clinical trial does not have a data and safety monitoring board (DSMB).

Informed consent

All patients will be informed by the investigator(s) of the aims of the study, the possible adverse events, the procedures, the possible hazards to which he/she will be exposed, who has access to their patient data and what provisions were made for compensating those who suffer harm from trial participation. It will be emphasized that the participation is voluntary and that the patient is allowed to refuse further participation in the protocol whenever he/she

wants. This will not prejudice the patient's subsequent care. Documented informed consent must be obtained for all patients included in the study before they are enrolled in the study.

Harms

(Serious) adverse events and (serious) adverse drug reactions will be collected and recorded throughout the study period, starting at day 1 of the treatment through 1 month after the last dose of investigational product in accordance with Good Clinical Practice guidelines as described in the International Conference on Harmonization Guideline (ICH-GCP). It will be left to the investigator's clinical judgment to determine whether an adverse event is related and of sufficient severity to require the subject's removal from treatment or from the study. A subject may also voluntarily withdraw from treatment. A potential harm for patients concerns overlapping side-effects of metformin and chloroquine, which are mainly of gastro-intestinal nature.

Ethics and dissemination

This study is being conducted according ICH-GCP and in accordance with general ethical principles outlined in the Declaration of Helsinki. Ethical approval was obtained from the medical-ethical committee of the Academic Medical Centre, Amsterdam (MEC-AMC), the Netherlands and the Dutch national competent authority (CCMO) on 22 October 2015 and 13 January 2016, respectively, under reference number NL53150.018.15. Informed consent forms were approved by the MEC-AMC.

A report describing the results of the study will be submitted to a peer-reviewed journal. Where permitted by patient data protection standards, data will be published and shared

together with the publication of the study results. Co-authorship will be based on standard International Committee of Medical Journal Editors (ICMJE) guidelines. No professional writers will be used.

An ethical limitation of the present clinical trial may be that the therapeutic index of metformin and chloroquine has been established in glioma and colorectal carcinoma cells,[16] but not in intrahepatic cholangiocarcinoma or chondrosarcoma models. However, this is primarily a dose-finding study. Follow-up phase II clinical trials will be rationally designed based on the pending evidence whether or not the efficacy of metformin and chloroquine treatment will be validated in model systems of other types of cancer by then.

LIST OF ABBREVIATIONS

αKG	alpha-ketoglutarate
AML	acute myeloid leukaemia
BBB	blood-brain barrier
b.i.d.	bis in die, two times a day
CTCAE	common terminology criteria for adverse events
ctDNA	circulating tumour DNA
D-2HG	*D*-2-hydroxyglutarate
DLT	dose-limiting toxicity

ETC	electron transport chain
IDH1/2	isocitrate dehydrogenase 1 or 2
$IDH1/2^{WT}$	IDH1/2 wild-type
$IDH1/2^{MT}$	IDH1/2 mutant
MAD	maximum administered dose
MRS	magnetic resonance spectroscopy
MS	mass spectrometry
MTD	maximum tolerated dose
NGS	next-generation sequencing
q.d.	quaque die, one a day
RANO	response assessment in neuro-oncology
RD	recommended dose (for a phase II clinical trial)
RECIST	response evaluation criteria in solid tumours
TCA cycle	tricarboxylic acid cycle

DECLARATIONS

Consent for publication

Not applicable.

Availability of data and material

Data sharing not applicable to this article as no datasets were generated or analysed during the current study.

Competing interests:

The authors declare no conflict of interest.

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Authors' contributions

R.J.M. and J.W.W. conceived and designed the study. R.J.S., M.Kh., M.E.v.L., M.Ko., J.A.M.B., J.V.M.G.B., R.A.A., H.J.K, H.W.M.v.L., C.J.F.v.N., W.P.V., H.G. and T.M.v.G. guided the study design. R.J.M., M.Kh. and M.W.A.C. performed pilot study experiments. R.J.M. wrote the manuscript. All authors read and approved the final manuscript.

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Registration:

This article was registered at ClinicalTrials.gov under identifier NCT02496741.

Study dates:

Date of study registration: 30 June 2015

Date of ethical approval: 22 October 2015

Date of first enrollment: 17 November 2015

Patients included as of 25 October 2015: 3.

Expected date of enrollment completion: Q4 2017.

Clinical trial protocol:

The clinical trial protocol with version number 1.2 (22 September 2015) received approval from the medical-ethical committee of the Academic Medical Centre, Amsterdam (MEC-AMC), the Netherlands and the Dutch national competent authority (CCMO) on 22 October 2015 and 13 January 2016, respectively.

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FIGURE LEGENDS

Figure 1. Cellular carbohydrate and glutamine metabolism showing the pathways in which wild-type IDH1 and IDH2 are functional. Isocitrate dehydrogenase 1, 2, and 3 (IDH1/2/3) catalyse the conversion of isocitrate to α-ketoglutarate (αKG) in the cytoplasm

and mitochondria, respectively, where IDH1 and IDH2 are NADPH-producing and IDH3 is NADH-producing. This reaction occurs in tricarboxylic acid (TCA) cycle(-like) metabolism and is fed by glucose influx and/or glutamine influx. The conversion of glutamine into αKG occurs in the glutaminolysis pathway and the last step is catalysed by glutamate dehydrogenase (GDH), which is inhibited by chloroquine and metformin. The TCA cycle produces NADH, which is used in the electron transport chain (ETC) and oxidative phosphorylation to generate ATP. Complex I of the ETC is inhibited by metformin. Wild-type IDH1/2 ($IDH1/2^{WT}$) differs from mutant IDH1/2 ($IDH1/2^{MUT}$) because the latter enzyme converts αKG into a novel oncometabolite, *D*-2-hydroxyglutarate (*D*-2HG).

Figure 2. Dosing schedule and study design for patients who will not undergo tumour resection. Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

Figure 3. Dosing schedule and study design for patients who have a tumour resection planned. Surgery will define the end of this time schedule (2 days before surgery). Abbreviations: *D*-2HG, *D*-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

TABLES AND LEGENDS

Table 1. Inclusion and exclusion criteria.

Key inclusion criteria
• Age ≥18 years. • Presence of a measurable intrahepatic cholangiocarcinoma or WHO grade II-III chondrosarcoma (RECIST 1.1 criteria [69]) or WHO grade II-IV glioma (RANO criteria [70]), both newly-diagnosed and refractory, relapsed, or recurrent tumours. • Tumour carries a *D*-2HG-generating mutation in *IDH1* or *IDH2* as determined by sequencing of primary tumour DNA, immunohistochemistry of primary tumour tissue with an IDH1/2 mutant-specific antibody, or MRS imaging of the tumour (for glioma patients). • ECOG/WHO performance status 0-2. • Adequate renal function (creatinine <150 µmol/L or a creatinine clearance >60 ml/L). • Adequate liver function (bilirubin <1.5 times the normal upper limit; ALAT and ASAT <2.5 the normal upper limit). • Adequate bone marrow function (white blood cells >3.0 x 10^9/L, platelets >100 x 10^9/L). • When patient is eligible for tumour resection, surgery is planned at least 4 weeks later than the start of study treatment.
Key exclusion criteria

• Concomitant other anti-cancer therapy (e.g. surgical resection, chemotherapy, targeted therapy, radiation therapy, surgery). Palliative therapy is permitted, such as:
  ○ palliative radiotherapy for symptomatic bone metastases,
  ○ dexamethasone for symptom relief in patients with glioma and cerebral oedema,
  ○ non-enzyme inducing anti-epileptic drugs (with the exception of topiramate) in patients with glioma and epileptic seizures.
• Severe and/or uncontrolled medical conditions at <6 months prior to randomization, such

as:

- unstable angina pectoris, symptomatic congestive heart failure, myocardial infarction or cardiac arrhythmias,
- pulmonary insufficiency,
- severe gastrointestinal, neurological (including epilepsy) or hematological diseases (interaction with chloroquine),
- uncontrolled diabetes as defined by fasting serum glucose >12 mmol/l,
- active or uncontrolled severe infection, including malaria,
- cirrhosis, chronic active hepatitis or chronic persistent hepatitis.

- Serious concomitant systemic disorder that compromises the safety of the patient, at the discretion of the investigator.
- Patients who have a known history of alcohol abuse (interaction with metformin).
- Patients with known glucose-6-phosphate dehydrogenase deficiency, porphyria, myasthenia gravis or ocular/retinal aberrations (interactions with chloroquine).
- Patients who use digoxin, MAO inhibitors, fenylbutazone, oxygenbutazone, gold preparations or cimetidine (known pharmacokinetic interactions with chloroquine) or loop diuretics (known pharmacokinetic interaction with metformin) for which not a good alternative is available.
- Patients with a known hypersensitivity to metformin or chloroquine.
- Use of metformin or chloroquine in the previous 6 months or long-term use of chloroquine (>5 years or cumulative dose >300 grams) in the past.

Abbreviations: ctDNA, circulating tumour DNA; *D*-2HG, *D*-2-hydroxyglutarate; IDH1/2, isocitrate dehydrogenase 1 and 2; MRS, magnetic resonance spectroscopy; MS, mass spectrometry.

Table 2. Metformin dose escalation schedule.

Dose level	Dose of metformin given orally (total daily dose)	Minimum number of patients
-1	500 mg q.d. (500 mg total)	--
1 (starting)	500 mg b.i.d. (1000 mg total)	3
2	1000 mg b.i.d. (2000 mg total)	3
3	1500 mg b.i.d. (3000 mg total)	3

Abbreviations: b.i.d., two times a day; q.d., once a day.

Table 3. Timeline, study treatment, study visits and medical procedures.

Required investigations	Screening	Day 8 (week 2)	Day 29/week 5 and every 4 weeks thereafter	Day 57/week 9 and every 8 weeks thereafter	End of study
Visit number	1	2	3	4+	4+
Written informed consent	Prior to Screening				
Demographics (age, sex)	X				
Overall medical history	X				X
Physical examination, including weight and height	X		X	X	X

Vital signs (blood pressure, pulse)	X		X	X	X
ECOG/WHO performance status	X		X	X	X
CT or MRI scan of measurable lesion, ≤1 month prior to start treatment	X			X	X
Haematology	X		X	X	X
Serum chemistry:					
Hepatic function	X		X	X	X
Renal function	X		X	X	X
Glucose	X		X	X	X
HbA1c	X			X	X
Triglycerides	X			X	X
Cholesterol	X			X	X
Haemostatic parameters (aPPT and PT)	X				X
Insulin, IGF-1, IGF binding protein-3	X			X	X
Vitamin B12	X			X	X

Metformin concentration		X	X	X	X
Chloroquine concentration			X	X	X
MS of serum/urine/bile for *D*-2HG levels	X		X	X	X
MRS for intratumoural 2HG levels	X				X
Liquid biopsy	X		X	X	X
ECG	X				
Pregnancy test	X				
Optional: tumour biopsy	X			X	X

In addition to this scheme, an ECG will be performed every 24 weeks. Metformin and chloroquine concentrations will be taken at the end of study only when possible.

Abbreviations: (*D*-)2HG, (*D*-)2-hydroxyglutarate; IGF, insulin growth factor; MRS, magnetic resonance spectroscopy; MS, mass spectroscopy.

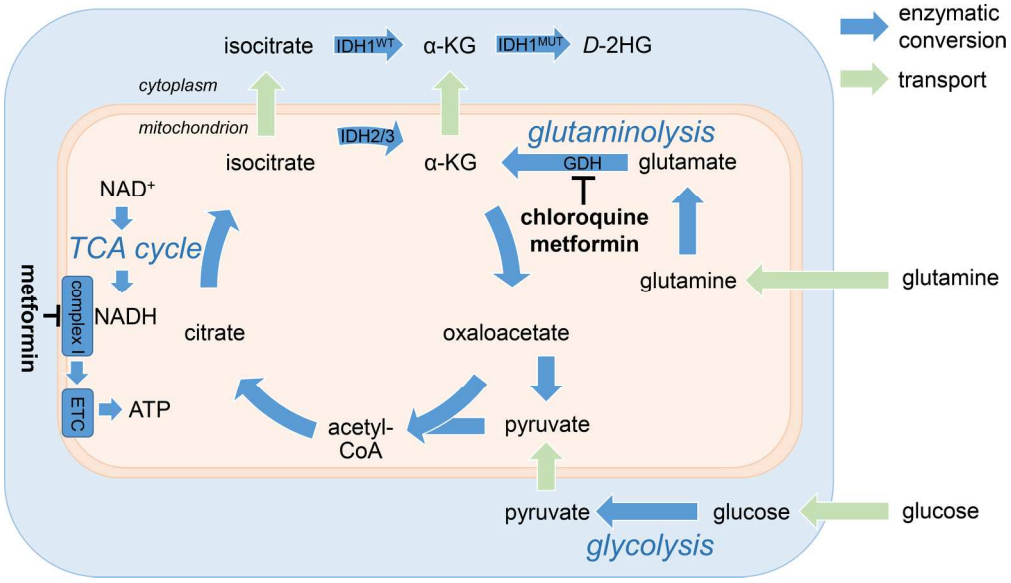

Figure 1. Cellular carbohydrate and glutamine metabolism showing the pathways in which wild-type IDH1 and IDH2 are functional. Isocitrate dehydrogenase 1, 2, and 3 (IDH1/2/3) catalyse the conversion of isocitrate to α-ketoglutarate (αKG) in the cytoplasm and mitochondria, respectively, where IDH1 and IDH2 are NADPH-producing and IDH3 is NADH-producing. This reaction occurs in tricarboxylic acid (TCA) cycle(-like) metabolism and is fed by glucose influx and/or glutamine influx. The conversion of glutamine into αKG occurs in the glutaminolysis pathway and the last step is catalysed by glutamate dehydrogenase (GDH), which is inhibited by chloroquine and metformin. The TCA cycle produces NADH, which is used in the electron transport chain (ETC) and oxidative phosphorylation to generate ATP. Complex I of the ETC is inhibited by metformin. Wild-type IDH1/2 (IDH1/2WT) differs from mutant IDH1/2 (IDH1/2MUT) because the latter enzyme converts αKG into a novel oncometabolite, D-2-hydroxyglutarate (D-2HG).

Figure 1
99x56mm (600 x 600 DPI)

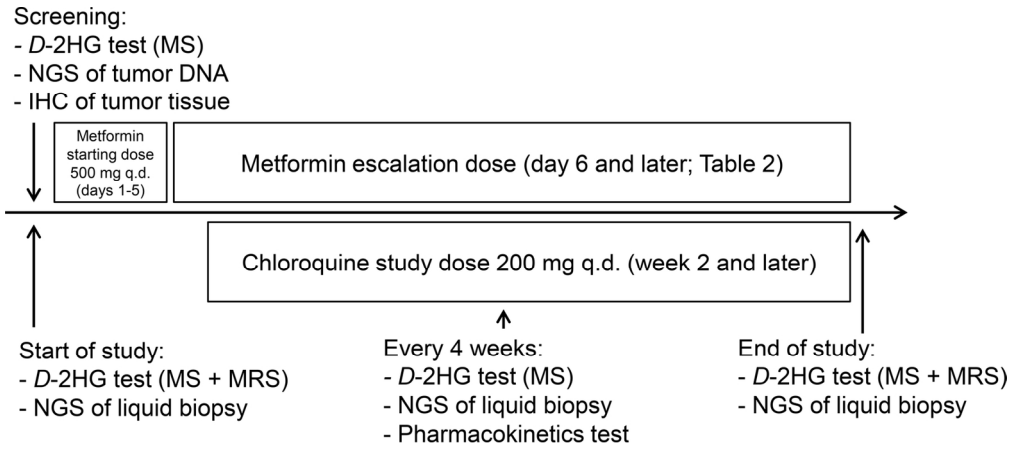

Figure 2. Dosing schedule and study design for patients who will not undergo tumour resection. Abbreviations: D-2HG, D-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.
Figure 2
75x32mm (600 x 600 DPI)

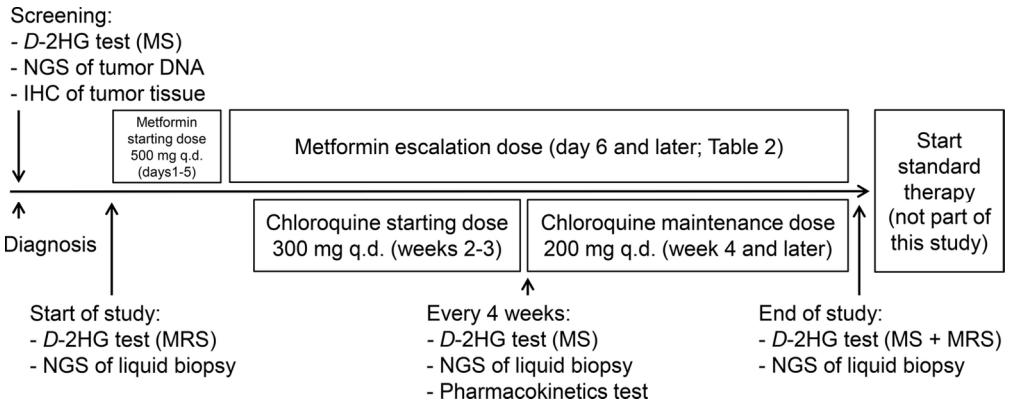

Figure 3. Dosing schedule and study design for patients who have a tumour resection planned. Surgery will define the end of this time schedule (2 days before surgery). Abbreviations: D-2HG, D-2-hydroxyglutarate; IHC, immunohistochemistry; MRS, magnetic resonance spectroscopy; MS, mass spectrometry; NGS, next-generation sequencing; q.d., once a day.

Figure 3
67x26mm (600 x 600 DPI)

Supplementary Table 1. Hematologic and non-hematologic dose-limiting toxicities.

Hematologic	Non-hematologic
• Absolute granulocyte count <0.5 x 10^9/l. • Febrile neutropenia (ANC <1.0 x 10^9/L, fever >38.5°C). • Platelets <25 x 10^9/l. • Bleeding due to thrombocytopenia, as determined by a physician.	• Diarrhoea > grade 3 despite optimal loperamide use. • Rash > grade 3 or grade 2 is medically concerning or unacceptable to the patient. • Other grade 3 effects considered to be treatment related. • Missing >7 days of treatment for toxicity reasons.

Grading of side effects is performed using CTCAE. Abbreviations: ANC, absolute neutrophil count.

SUPPLEMENTARY FILE 1

3+3 dose-escalation schedule and intrapatient dose-escalation

Maximum administered dose

If 0/3 patients exhibit dose-limiting toxicity at this dose level:

- Dose escalation to the next dose level may begin in a new cohort of patients
- Patients enrolled on the previous dose level who are still receiving therapy may now undergo intrapatient dose escalation to this new dose level provided that they have experienced no drug related toxicity of grade 2 or higher at the previous dose level.

If 1/3 patients exhibit dose-limiting toxicity at this dose level:

- Expand dose level to a total of six patients. Toxicity information from patients who underwent intrapatient dose escalation can be used for expansion cohorts, but only when they have completed at least 8 weeks of treatment at the new dose level.
- If no further DLT events are observed, dose escalation to the next dose level may begin in a new cohort of patients and patients enrolled on the previous dose level who are still receiving therapy may now undergo intrapatient dose escalation to this dose level provided that they have experienced no drug related toxicity of grade 2 or higher.
- If further DLTs are observed (i.e. in ≥2/6 patients), this dose level will be considered the maximum administered dose (MAD).

If ≥2/3 patients exhibit dose-limiting toxicity

- This dose level will be considered the MAD.
- If this toxicity occurs at level 1 (starting level), dose de-escalation to level -1 will be applied.

Recommended phase II dose

As described in the full text manuscript, the MAD is the dose in which ≥2/3 or ≥2/6 patients experience a DLT, or the final dose from the dose escalation schedule (1500 mg metformin b.i.d. and 200 mg chloroquine q.d.). One dose level below the MAD will be considered the RD for follow-up phase II clinical trials. When the starting dose level (“1”) is the MAD, we will de-escalate the dose level to dose level “-1”. When we do not observe DLTs in three patients or one DLT in six patients at this dose level, then dose level “-1” will be the RD. When we observe more than one DLT, the combination of metformin and chloroquine will be considered too toxic to be useful in cancer patients. In contrast to this situation where we have to accept the lowest dose-escalation level as the RD, when 0/6 patients experience DLTs at the final dose level of the dose escalation schedule (i.e. dose level “3”), this can be considered the RD for follow-up phase II clinical trials, instead of dose level “2”.

Up to a total of six patients may be treated at the RD level to assure information on the safety profile when that dose is complete. When clinically appropriate, intermediate dose levels may be studied to assure that the RD is the highest tolerable. Furthermore, when pharmacokinetic data suggests that saturating absorption of drug is occurring on a b.i.d. oral administration

level, further dose splitting to three times a day or four times a day schedules may be considered.

Patient replacement

Three patients within a dose level must be observed for eight weeks before accrual to the next dose level may begin. If a patient is withdrawn from the study prior to completing 22 days of therapy without experiencing a DLT prior to withdrawal, an additional patient may be added to that dose level. Patients missing seven or more doses (one week) due to toxicity will not be replaced since these patients will be considered to have experienced a dose-limiting toxicity.

Data monitoring

Based on the guideline by the NFU (Dutch Federation of University Medical Centers) about quality insurance in human research ("Kwaliteitsborging van mensgebonden onderzoek") and the "Risk assessment in clinical research projects regarding the required management and monitoring strategy" by the AMC Clinical Research Unit, the risk of this study was qualified as 'moderate'.

According to this moderate risk a 'minimal intensive monitoring' is advised, which will be performed by an independent clinical research and consists of:

- 1 visit per year, per center
- 1-10% of patient cases will be checked for informed consent
- First 3 patients per center will be checked on in/exclusion criteria, then 1-10% of patients thereafter
- 1-10% of patient cases will be checked for Source Data Verification
- 1-10% of patients will be checked for SAEs (serious adverse events) and SADRs (serious adverse drug reactions)

SPIRIT 2013 Checklist: Recommended items to address in a clinical trial protocol and related documents*

Section/item	Item No	Description	Addressed on page number
Administrative information			
Title	1	Descriptive title identifying the study design, population, interventions, and, if applicable, trial acronym	1
Trial registration	2a	Trial identifier and registry name. If not yet registered, name of intended registry	26
	2b	All items from the World Health Organization Trial Registration Data Set	
Protocol version	3	Date and version identifier	26
Funding	4	Sources and types of financial, material, and other support	25
Roles and responsibilities	5a	Names, affiliations, and roles of protocol contributors	1,25
	5b	Name and contact information for the trial sponsor	1
	5c	Role of study sponsor and funders, if any, in study design; collection, management, analysis, and interpretation of data; writing of the report; and the decision to submit the report for publication, including whether they will have ultimate authority over any of these activities	25
	5d	Composition, roles, and responsibilities of the coordinating centre, steering committee, endpoint adjudication committee, data management team, and other individuals or groups overseeing the trial, if applicable (see Item 21a for data monitoring committee)	N/A

Introduction			
Background and rationale	6a	Description of research question and justification for undertaking the trial, including summary of relevant studies (published and unpublished) examining benefits and harms for each intervention	4-11
	6b	Explanation for choice of comparators	4-11
Objectives	7	Specific objectives or hypotheses	11-14
Trial design	8	Description of trial design including type of trial (eg, parallel group, crossover, factorial, single group), allocation ratio, and framework (eg, superiority, equivalence, noninferiority, exploratory)	11, 14-16
Methods: Participants, interventions, and outcomes			
Study setting	9	Description of study settings (eg, community clinic, academic hospital) and list of countries where data will be collected. Reference to where list of study sites can be obtained	12
Eligibility criteria	10	Inclusion and exclusion criteria for participants. If applicable, eligibility criteria for study centres and individuals who will perform the interventions (eg, surgeons, psychotherapists)	Table 1 (page 32-33)
Interventions	11a	Interventions for each group with sufficient detail to allow replication, including how and when they will be administered	15-16, Table 2-3 (page 34-36)
	11b	Criteria for discontinuing or modifying allocated interventions for a given trial participant (eg, drug dose change in response to harms, participant request, or improving/worsening disease)	15-17, 22
	11c	Strategies to improve adherence to intervention protocols, and any procedures for monitoring adherence (eg, drug tablet return, laboratory tests)	N/A
	11d	Relevant concomitant care and interventions that are permitted or prohibited during the trial	Table 1 (page 32)
Outcomes	12	Primary, secondary, and other outcomes, including the specific measurement variable (eg, systolic blood pressure), analysis metric (eg, change from baseline, final value, time to event), method of aggregation (eg, median, proportion), and time point for each outcome. Explanation of the clinical relevance of chosen efficacy and harm outcomes is strongly recommended	14-15
Participant timeline	13	Time schedule of enrolment, interventions (including any run-ins and washouts), assessments, and visits for participants. A schematic diagram is highly recommended (see Figure)	Table 3 (page 34-36), Figure 2-3

Sample size	14	Estimated number of participants needed to achieve study objectives and how it was determined, including clinical and statistical assumptions supporting any sample size calculations	15, 20-21
Recruitment	15	Strategies for achieving adequate participant enrolment to reach target sample size	N/A
Methods: Assignment of interventions (for controlled trials)			
Allocation:			
Sequence generation	16a	Method of generating the allocation sequence (eg, computer-generated random numbers), and list of any factors for stratification. To reduce predictability of a random sequence, details of any planned restriction (eg, blocking) should be provided in a separate document that is unavailable to those who enrol participants or assign interventions	N/A
Allocation concealment mechanism	16b	Mechanism of implementing the allocation sequence (eg, central telephone; sequentially numbered, opaque, sealed envelopes), describing any steps to conceal the sequence until interventions are assigned	NA
Implementation	16c	Who will generate the allocation sequence, who will enrol participants, and who will assign participants to interventions	N/A
Blinding (masking)	17a	Who will be blinded after assignment to interventions (eg, trial participants, care providers, outcome assessors, data analysts), and how	N/A
	17b	If blinded, circumstances under which unblinding is permissible, and procedure for revealing a participant's allocated intervention during the trial	N/A
Methods: Data collection, management, and analysis			
Data collection methods	18a	Plans for assessment and collection of outcome, baseline, and other trial data, including any related processes to promote data quality (eg, duplicate measurements, training of assessors) and a description of study instruments (eg, questionnaires, laboratory tests) along with their reliability and validity, if known. Reference to where data collection forms can be found, if not in the protocol	Table 3 (page 34-36)
	18b	Plans to promote participant retention and complete follow-up, including list of any outcome data to be collected for participants who discontinue or deviate from intervention protocols	Table 3 (page 34-36)

Data management	19	Plans for data entry, coding, security, and storage, including any related processes to promote data quality (eg, double data entry; range checks for data values). Reference to where details of data management procedures can be found, if not in the protocol	21
Statistical methods	20a	Statistical methods for analysing primary and secondary outcomes. Reference to where other details of the statistical analysis plan can be found, if not in the protocol	20-21
	20b	Methods for any additional analyses (eg, subgroup and adjusted analyses)	N/A
	20c	Definition of analysis population relating to protocol non-adherence (eg, as randomised analysis), and any statistical methods to handle missing data (eg, multiple imputation)	N/A
Methods: Monitoring			
Data monitoring	21a	Composition of data monitoring committee (DMC); summary of its role and reporting structure; statement of whether it is independent from the sponsor and competing interests; and reference to where further details about its charter can be found, if not in the protocol. Alternatively, an explanation of why a DMC is not needed	21
	21b	Description of any interim analyses and stopping guidelines, including who will have access to these interim results and make the final decision to terminate the trial	N/A
Harms	22	Plans for collecting, assessing, reporting, and managing solicited and spontaneously reported adverse events and other unintended effects of trial interventions or trial conduct	22
Auditing	23	Frequency and procedures for auditing trial conduct, if any, and whether the process will be independent from investigators and the sponsor	21
Ethics and dissemination			
Research ethics approval	24	Plans for seeking research ethics committee/institutional review board (REC/IRB) approval	3, 22
Protocol amendments	25	Plans for communicating important protocol modifications (eg, changes to eligibility criteria, outcomes, analyses) to relevant parties (eg, investigators, REC/IRBs, trial participants, trial registries, journals, regulators)	N/A

Consent or assent	26a	Who will obtain informed consent or assent from potential trial participants or authorised surrogates, and how (see Item 32)	21-22
	26b	Additional consent provisions for collection and use of participant data and biological specimens in ancillary studies, if applicable	N/A
Confidentiality	27	How personal information about potential and enrolled participants will be collected, shared, and maintained in order to protect confidentiality before, during, and after the trial	21-22
Declaration of interests	28	Financial and other competing interests for principal investigators for the overall trial and each study site	25
Access to data	29	Statement of who will have access to the final trial dataset, and disclosure of contractual agreements that limit such access for investigators	21-22
Ancillary and post-trial care	30	Provisions, if any, for ancillary and post-trial care, and for compensation to those who suffer harm from trial participation	21-22
Dissemination policy	31a	Plans for investigators and sponsor to communicate trial results to participants, healthcare professionals, the public, and other relevant groups (eg, via publication, reporting in results databases, or other data sharing arrangements), including any publication restrictions	23
	31b	Authorship eligibility guidelines and any intended use of professional writers	22
	31c	Plans, if any, for granting public access to the full protocol, participant-level dataset, and statistical code	N/A
Appendices			
Informed consent materials	32	Model consent form and other related documentation given to participants and authorised surrogates	N/A
Biological specimens	33	Plans for collection, laboratory evaluation, and storage of biological specimens for genetic or molecular analysis in the current trial and for future use in ancillary studies, if applicable	17-19

*It is strongly recommended that this checklist be read in conjunction with the SPIRIT 2013 Explanation & Elaboration for important clarification on the items. Amendments to the protocol should be tracked and dated. The SPIRIT checklist is copyrighted by the SPIRIT Group under the Creative Commons "Attribution-NonCommercial-NoDerivs 3.0 Unported" license.